DOCTOR OF PHILOSOPHY

In vitro osteoclast resorption of calcium phosphate bone substitutes

Martin, Joanne

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In vitro osteoclast resorption of calcium phosphate bone substitutes

Joanne Martin BSc, MSc

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"Life Is the Greatest Philosophy"

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Summary

The broad aim of this work was to investigate the *in vitro* osteoclast (OC) resorption of calcium phosphate (CaP) biomaterials. Resorption of CaP biomaterials is traditionally assessed using an OC resorption assay where resorption pits formed on the CaP surface are analysed by microscopy techniques and quantified on the basis of pit number, pit area or pit volume. Apart from the time consuming nature of the analysis techniques, there are many limitations associated with the current methods. Pit area measurements (2D) have become common practice when assessing CaP biomaterial resorption *in vitro* but it is not a precise indicator of resorption; variations in pit depth are not taken into consideration and it is unsuitable for use on porous materials where visualisation of internal structures is difficult or for use on materials with rough surfaces where determination of individual pits would be difficult. A 3D quantification of bioresorption is available but requires specialised, expensive equipment.

An appropriate measure of resorption was required to be more efficient and more cost-effective than the current available *in vitro* methods, but most importantly, to directly correlate with pit area measurements and have the ability to be used in a broad range of *in vitro* experiments. Thus, the aim of this work was to establish the suitability of several outcome measures as indicators of OC resorption *in vitro* that directly correlate with the current pit area measurements.

An OC resorption assay was developed for the generation of actively resorbing OC *in vitro*. Several experiments were conducted in order to optimise the resorbability of the generated OC. Cell culture variables included: cell type, OC differentiation factor concentration, media type, cell seeding density, pH and cell culture period.

Once the OC assay was established several outcome measures were assessed as potential indicators of resorption *in vitro*, namely; the correlation of percentage area resorbed *in vitro* with Ca and P mineral release into cell culture medium, OC number

and OC activity. In order to accurately correlate pit area with the alternative outcome measures the assay was carried out using dense beta-Tricalcium Phosphate (β-TCP). The rationale for using a dense substrate was two-fold. Firstly, it allowed pit formation and area to be analysed devoid of material anomalies or pores, which are frequently observed with less dense substrates and can give rise to discrepancies within results. Secondly, the current 2D methods for measuring resorption perform best on a substrate free from imperfections.

This body of work has established two main outcome measures of bioresorption *in vitro* that correlate with pit area measurements; Ca and P mineral release into the culture medium and OC specific enzyme activity. Both outcome measures assess bioresorption using biochemical techniques. These outcome measures have shown potential as indicators of bioresorption *in vitro* and will prove invaluable for improving the fundamental understanding of OC resorption of CaP biomaterials.

Dissemination of Information

Conference Publications

- **Bioengineering in Ireland 2012**
  "*Possible outcome measures as indicators for osteoclast resorption on β-TCP*"

- **European Society for Biomaterials 2011**
  "*A comparison of Osteoclast Resorption on Calcined and non-Calcined β-TCP*"

- **Northern Ireland Biomedical Engineering Society 2011**
  "Osteoclast Resorption on Thermally Treated β-TCP"

- **Northern Ireland Biomedical Engineering Society 2010**
  "Outcome measures for the cell mediated resorption of calcium phosphate bone substitutes"

- **Bioengineering in Ireland 2010**
  "Cell mediated bioresorption of calcium phosphate bone substitutes"

Contents

ABBREVIATIONS USED

ANOVA	One-way Analysis of Variance
ATP	Adenosine Triphosphate
BCP	Biphasic Calcium Phosphate
BSA	Bovine Serum Albumin
Ca	Calcium
Ca^{2+}_{i}	Intracellular Calcium
CaP	Calcium Phosphate
CC	Calcium Carbonate
CF	Cell Free
CO_2	Carbon Dioxide
DCP	Dicalcium Phosphate
DCPD	Dicalcium Phosphate Dihydrate
D-MEM	Dulbecco's Modified Eagles Medium
ECa^{2+}	Extracellular Calcium
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Foetal Bovine Serum

HA	Hydroxyapatite
H_2CO_3	Carbonic Acid
H_2O	Water
$HCO3^-$	Bicarbonate ion
HCl	Hydrochloric Acid
HMDS	Hexamethlysilasane
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ISO	International Standard
LM	Light Microscopy
M	Mass after drying
MCPM	Monocalcium Phosphate
M-CSF	Macrophage Colony-Stimulating Factor
MMP	Metalloproteinase
M_o	Initial Mass
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
OB	Osteoblast
OC	Osteoclast

OCP	Octocalcium Phosphate
OPC	Osteoclast Precursor Cell
OPG	Osteoprotegerin
P	Phosphate
PBS	Phosphate Buffered Saline
PCL	Poly ε-Caprolactone
PGA	Polyglycolide
PLA	Polylactide
pNP	p-Nitrophenol
pNPP	p-Nitrophenol Phospahte
PMMA	Polymethylmethacrylate
RANKL	Receptor Activator for activation of nuclear factor κβ-Ligand
RGD	Arginine-Glycine-Aspartic Acid
RLM	Reflected Light Microscopy
RMS	Robert Mathy's Foundation
SA	Surface Area
SEM	Scanning Electron Microscopy
SiC	Silicon Carbide

TCP	Tri-Calcium Phosphate
TRAP	Tartrate-Resistant Acid Phosphatase
XRD	X-ray Diffraction
XTT	Tetrazolium salt
ZnTCP	Zinc Substituted Tricalcium Phosphate
α-MEM	Alpha Modification Minimum Essential Medium

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CHAPTER ONE

Introduction

1 Introduction

The field of biomaterials is interdisciplinary, requiring knowledge and expertise from many backgrounds including; material scientists, cell biologists and mechanical engineers. Biomaterials are relevant to many areas of research such as tissue engineering, regenerative medicine and medical devices and implants. Amongst these, orthopaedic implants are the most common body implant [1] with metal, polymeric and ceramic materials used in such devices.

Research direction is strongly influenced by commercial markets. The Global biomaterials market was valued at $28.8 billion in 2009 and is estimated to rise to $58.1 billion by 2014 [1]. $3.5 billion of this global market was attributed to the US orthopaedic biomaterials market, which is estimated to reach $6.3 billion by 2016 [2]. Such growth can be attributed to the demographic profile of an ageing population and the prevalence of osteoarthritis; however, with an increased awareness of the benefits of physical activity more people are physically active into their later years. This increases the need for health maintenance, specifically joint care, as such activity is often the cause of fractures, degenerative disc disease and hip and knee problems.

Bone grafting using autologous (patient's) bone is the 'gold standard' [3,4]. Allograft (donor) bone is also used but there are associated limitations with both autograft and allograft; a second surgical procedure with related donor-site morbidity (autografts), problems with immunogenicity and demand outweighing supply (allografts). This has led to a demand for synthetic bone grafts [5] but to date commercially available synthetic grafts have been unable to match the clinical results seen with autograft [3].

Current research related to synthetic bone graft development is focused on more biocompatible and bioactive materials compared to the metallic implants previously used for fixation. The two main reasons for this shift are i) to improve patient recovery with the elimination of a retrieval operation and ii) to reduce healthcare expenses [5]. However, metals such as stainless steel, cobalt-chrome and titanium are still required in joint replacement and fracture fixation for their mechanical properties.

Ideally synthetic bone grafts should be biocompatible, integrate with the bone resorption process and aid new bone ingrowth whilst retaining sufficient mechanical strength. Throughout the last decade resorbable bone substitutes have been introduced in a bid to match, if not exceed, the clinical results seen with autograft [6]. Resorbable materials which can utilise the bones natural remodelling process to degrade, releasing non-toxic by-products that can be easily metabolised by the body, are very attractive for use as bone graft substitutes.

1.1 Bone structure

Bone is a dynamic tissue and has important functions beyond simply structural support. Bone provides protection to bodily organs, is the primary site for haemopoiesis and plays a role in mineral homeostasis, with calcium as its primary metabolite [6].

1.1.1 Bone matrix

Bone is a connective tissue composed of extracellular matrix and water with a 9:1 weight ratio respectively [7]. The extracellular matrix can be sub-divided into organic and inorganic phases. The organic phase consists of several protein families; collagens, proteoglycans and glycoproteins. Type 1 collagen makes up 90% of bone

protein with the remaining 10% consisting of smaller non-structural proteins, growth factors and blood proteins that assist bone mineralisation [8]. The inorganic phase is mostly microcrystalline HA but also contains some trace elements such as sodium, magnesium and fluoride [7]. During mineralisation HA crystals develop between the long collagen fibril axes to form a highly specialised tissue displaying properties of high stiffness, strength and flexibility [7].

1.1.2 Structural forms:

There are two macroscopic types of adult (lamellar) bone; cortical and cancellous also known as compact and spongy bone respectively (Figure 1.1). Cortical bone is dense and represents ~80% of skeletal weight. This hard compact bone has a high density, giving it the ability to resist bending. Cancellous bone is less dense and forms a honey-comb type network that supports the distribution of mechanical load, resisting compression, through a series of orientated trabeculae (small struts). Trabeculae are arranged in accordance to Wolff's Law [8][9], with thickest trabeculae arranged in the direction of greatest stress.

Cortical bone is a complex system made up from structures called osteons. Osteons consist of cylinders of concentric lamellae that are orientated parallel to the long axis of bone. Cement lines, which lie parallel with the lamellae, define the outer edge of each osteon. In the centre of each osteon is a matrix free canal - the Haversion canal, which houses blood vessels and nerves essential for bone maintenance. Active osteons communicate with each other and the bone marrow cavity through Volkmann's canals, which link central Haversion canals of adjacent osteons (Figure 1.2). Irregular areas between individual osteons are known as interstitial lamellae and represent previously remodelled osteons [8].

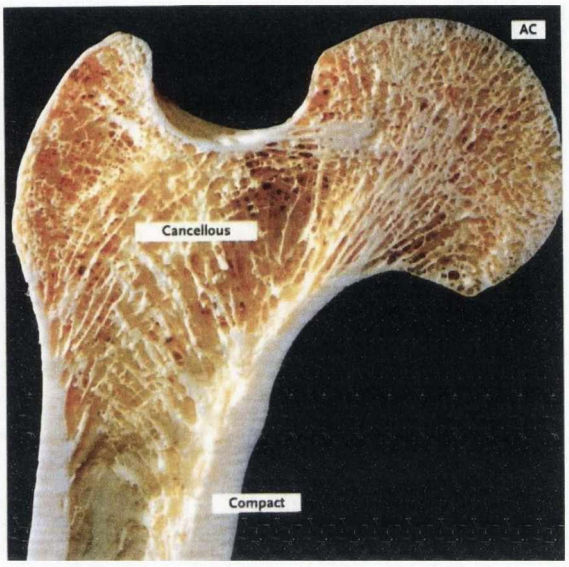

Figure 1.1: Macroscopic architecture of adult femur. Section through adult femur showing cortical (compact) and cancellous bone with a thin layer of articular cartilage (AC) covering the head of the femur [8]

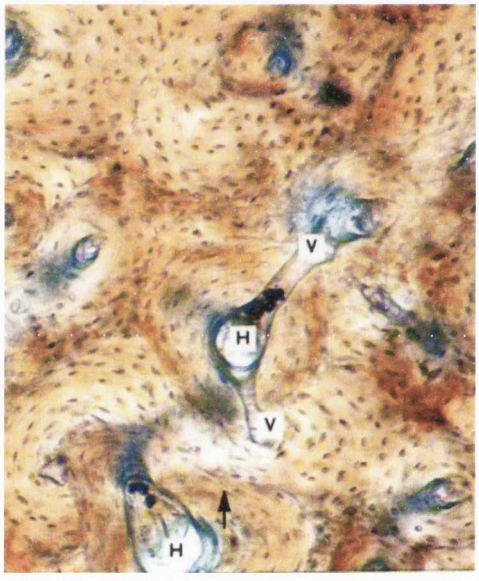

Figure 1.2: Unstained slice of cortical bone after formalin fixation. Transverse section through cortical bone shows osteons (groups of concentric cylinders) with central Haversion canals (H), linked by Volkmann's canals (V). Arrow denotes curving osteons [8].

1.1.3 Cellular components:

There are four main cell types associated with bone; osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts, which are classified by their location and function.

Osteoprogenitor cells are derived from mesenchymal stem cells found in the periosteum and bone marrow. Osteoprogenitor cells are capable of differentiating into specialised bone forming cells called osteoblasts (OB). When bone is not being formed these cells are dormant and are known as bone-lining cells, lining the internal and external surfaces of bone, known as the endosteum and periosteum respectively, with a single layer of flattened cells [8][10].

Osteoblasts (OB) also line the periosteal and endosteal surfaces of bone but display a cuboidal shaped cell layer. The role of the OB is to make new bone when required by secreting unmineralised bone matrix (osteoid) [8]. Active OB express high levels of alkaline phosphatase (an enzyme that catalyses the removal of bound phosphate groups by hydrolysis) which is thought to be an indicator of mineralisation by the liberation of phosphate ions into the bone matrix [10].

During bone formation some OB cells become trapped within the mineralised matrix and differentiate into osteocytes. These engulfed osteocytes form an interconnected network via a series of small tunnels called canaliculi, throughout newly formed lamellar bone. Osteocyte cytoplasmic processes extend through the canaliculi and allow communication between individual osteocytes [8] (Figure 1.3). When bone is under mechanical stress, osteocytes are thought to act as stress sensors that mediate the bones response to stress by signalling to OB and osteoclasts (OC) [10].

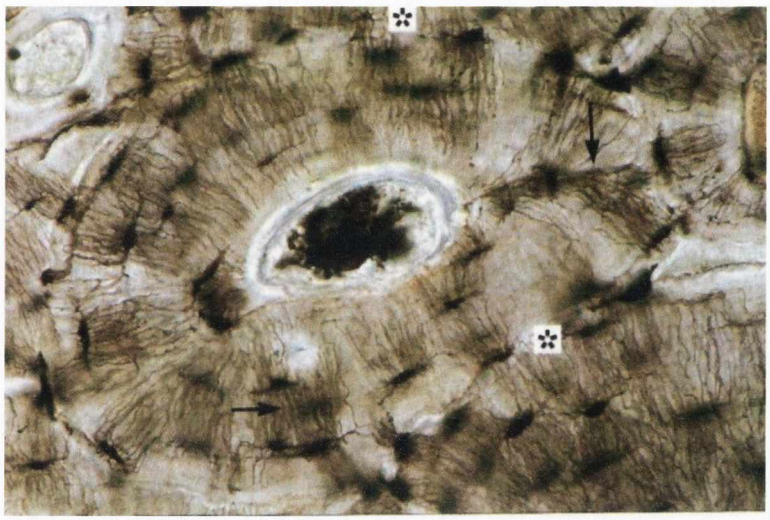

Figure 1.3: Transverse section of an osteon at high magnification. Arrow indicates communicating cytoplasmic extensions between individual osteocytes. Canaliculi carry cytoplasmic extensions between osteocytes allowing communication with adjacent osteons(*) [8].

OC are large multinucleated phagocytic cells derived from hematopoietic precursors present in bone marrow and the peripheral circulation [12]. OC have typical morphological features that help distinguish them from other bone cells such as multiple nuclei, a polarised apico-basal membrane and a highly ruffled membrane (ruffled-border) when actively attached to bone [12] . OC are highly motile and do not actively resorb until they anchor onto bone where they then secrete acid (to dissolve the inorganic phase) and proteolytic enzymes (to break down the organic matrix) forming characteristic areas of resorbed matrix, known as resorption pits or Howship's Lacunae [10]. Active OC express high levels of tartrate-resistant acid phosphatase (TRAP) which is believed to be related to their resorbing activity [13].

1.1.4 Bone remodelling:

Adult bone matrix continually undergoes a process of repair and renewal by OC and OB. In healthy adults bone remodelling is a normal homeostatic mechanism, resulting in a relatively stable bone mass. When this balance is disturbed by pathological conditions and disease the number of OC, the number of resorption sites and/or the rate of bone formation is altered and can result in an under- or over-production of bone [12]. There are many conditions that result in net bone loss; osteoporosis, rheumatoid arthritis, periodontitis and tumour-induced osteolysis to name a few [10].

The current concept of bone remodelling consists of four distinct phases [4], (Figure 1.4);

1) Resorptive phase: multinucleated OC resorb mineralised bone matrix
2) Reversal phase: osteoprogenitor cells, which differentiate into OB, migrate to the site of resorption
3) Formative phase: OB deposit unmineralised osteoid into the resorptive pit
4) Resting phase: once embedded in osteoid, OB differentiate into mature osteocytes and the bone matrix becomes mineralised. Lining cells cover the surface of the newly formed bone until the remodelling cycle starts again.

The remodelling cycle occurs within small groups of cell units called basic multicellular units (BMUs). At any given time ~20% cancellous bone is being remodelled [4]; however the rate of bone turnover is determined by the overall rate of both resorption and formation. The resorption phase may take up to 4 weeks whereas bone formation can take up to 6 months [11].

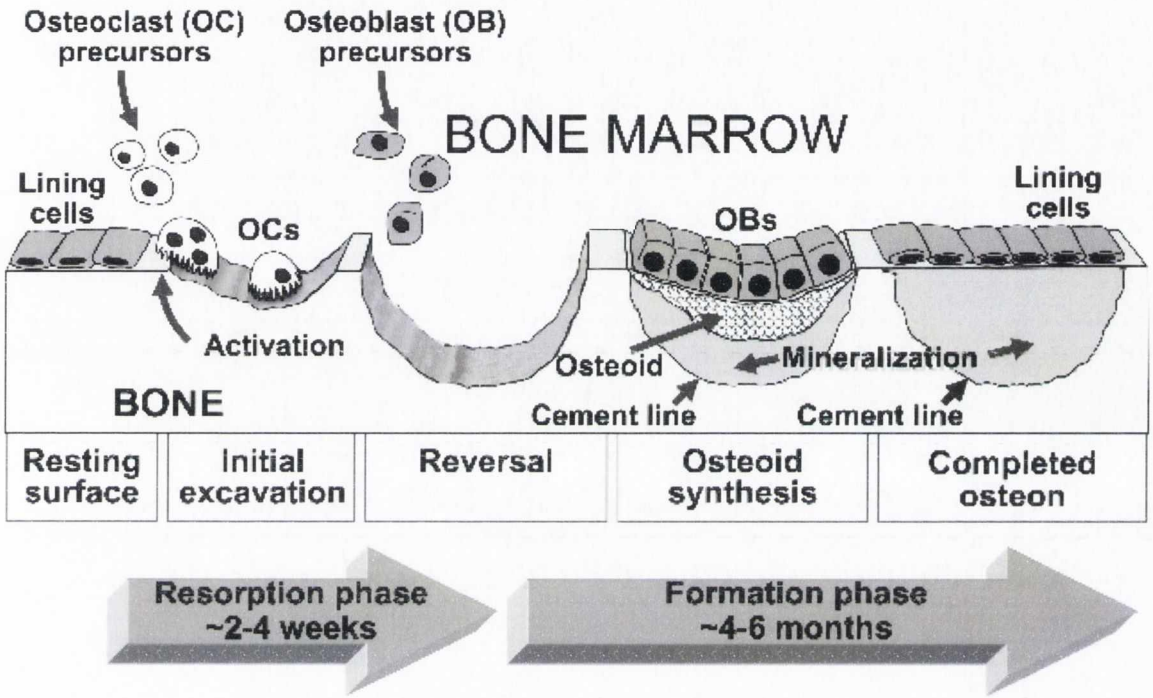

Figure 1.4: Bone remodelling cycle. This figure shows the remodelling phases; resorptive, reversal, formative and resting and the subsequent cell type associated with each phase[11].

1.2 Materials used for bone reconstruction

The two main events that can disturb bone haemostasis are disease and trauma. In cases of significant trauma, damage may be too extensive for natural bone remodelling to occur and implant surgery is the most likely treatment option, often in conjunction with a bone graft to stimulate healing [12].

1.2.1 Synthetic orthopaedic biomaterials

Metals

Metals have long been used as biomaterials in orthopaedics due to their excellent mechanical properties and easy availability. Metals have generally been considered to provide good clinical results with regards to fracture healing but they are not without their drawbacks. Metal implants such as stainless steel have a much higher Young's Modulus (210 GPa) compared to that of bone (10-20 GPa) [13]. It is this rigid, high stiffness property of the implant that allows it to carry load during healing, however, this causes the growing bone to carry insufficient load which can weaken it and subsequently lead to re-fracture after removal of the metal implant [13]. This happens in accordance with Wolff's Law where increasing the applied load to bone stimulates remodelling over time thus promoting bone growth whereas depriving the bone from loading will subsequently weaken the bone due to a lack of stimulus for remodelling and hence a reduction in bone mass. Therefore, if the metal implant is taking the entire load, growing bone is shielded from the load transmission during healing, a process known as stress-shielding. Other problems with metal implants include an increased sensitisation of the surrounding soft tissue and the requirement of a removal operation.

Polymers

Polymeric materials can be classified according to two parameters; 1) natural or synthetic and 2) biodegradable or non-biodegradable. The main advantage of using biodegradable polymeric materials is that they don't require a second removal operation. Synthetic biodegradable polymers show properties that make them

particularly suited for non-permanent orthopaedic implants as they can be tailored to produce a range of properties in comparison to natural biodegradable polymers[13]. In effect, synthetic biodegradable polymers can be designed to limit stress shielding, as they can be tailored to degrade at a rate proportional to the rate of bone growth transferring the load more effectively to the growing bone during healing[13]. Many biodegradable polymers are also referred to as bioabsorbable polymers. Both biodegradable and bioabsorbable polymers are biocompatible polymers that dissolve in the body after the healing process is complete. The difference is that biodegradable polymer by-products remain within the tissues long-term whereas bioabsorbable polymer by-products are either excreted or metabolised by the body.

Of all synthetic bioabsorbable polymers, aliphatic polyesters are considered to be the most important group due to their good biocompatibility, variable biodegradability and mechanical strength [14]. Some common aliphatic polyesters are polyglycolide (PGA), polylactide (PLA) and poly ε-caprolactone (PCL). These bioabsorbable polymers have been used for wound closure in the form of sutures and staples, for orthopaedic fixation devices such as pins and screws and as scaffolding materials for bone tissue engineering [14] however they are not without their drawbacks. Degradation products produced by PLA and PGA increase acid build up at the site of implantation which discourages OB activity and bone formation in turn leading to fibrous encapsulation of the implant [15]. Osteogenic bioceramics such as the calcium phosphate, hydroxyapatite (HA) have been incorporated into PLA in an attempt to increase the biocompatibility of the polymer in order for bone to form on the surface of the implant [16].

Ceramics

With HA accounting for 60% volume of the mineral phase of bone, calcium phosphate (CaP) bioceramics are natural choices as bone substitute materials and there has been extensive research into the use of HA and related CaPs as bone substitute and scaffolding materials [12][17]. CaP ceramics have enhanced osteointegrative and osteoconductive properties compared to metallic or polymeric materials [18]. Osseointegration is the ability to form a bond with the host bone material directly without interposition of fibrous tissue whereas osteoconduction is the ability to support new bone growth and vascularisation [12][19]. Some CaPs are also oseteoinductive [20] . Osteoinduction is where undifferentiated progenitor cells are recruited and stimulated to develop into preosteoblasts to induce osteogenesis.

CaP ceramics are either synthetic or natural in origin. Natural CaP materials are obtained from banked-bone or from naturally derived sea coral (coralline HA) [21]. Synthetic CaP materials can be divided into two groups based on their final synthesis route; aqueous precipitation and sintering. Many sintered ceramics are first produced by aqueous precipitation. CaP cements are produced by precipitation whereas most other CaP products are produced by sintering at high temperatures [21] [22]. CaP biomaterials have successfully been used in cranio-maxillofacial, orthopaedic and dental surgery [21], with β-TCP, HA and biphasic calcium phosphates (BCP) the traditional materials of choice. A detailed comparison of CaP ceramics used in medicine can be found in Bohner (2000) [22].

Most bone graft substitutes available for clinical use are based on CaP materials however clinical results remain inferior to those seen with autograft and allograft as CaP materials are not osteoinductive [3]. Therefore development of modified CaPs is required to further optimise their use.

1.3 Thesis Scope

This research was set up to investigate OC resorption of CaP biomaterials *in vitro*.

In vivo degradation of CaP ceramics occurs through a combination of cell mediated OC resorption and passive dissolution. In order to improve on the fundamental understanding of OC resorption of CaP in *vitro* a number of parameters must be considered; how does OC resorption occur, how can it be optimised and how can it be quantified *in vitro*?

OC attach to the implant surface and dissolve CaP through acid secretion and enzymatic action. When designing a new device for implantation, understanding the rate of resorption of the implant is a key factor. To be able to understand the rate of resorption, resorption at any given time must be quantified. This body of work will focus on quantifying OC resorption of CaP *in vitro*. OC resorption will be investigated through the development of an OC assay, optimised through a series of cell culture experiments and quantified through the assessment of various potential outcome measures *in vitro*. Outcome measures will be assessed for suitability as indicators of OC resorption *in vitro* and compared to current routine measures of resorption in a bid to provide an *in vitro* quantitative measure(s) of resorption that can be used in a variety of experimental conditions.

Hypothesis:

A simple, cost effective osteoclast resorption assay can be developed that will be more widely applicable to different ceramic materials and surfaces and enable prediction of *in vivo* resorption.

CHAPTER TWO

Literature Review

2 Literature review

2.1 Osteoclast biology and bone resorption

Osteoclastogenesis is the term used to describe the development of mature OC. It is a complex process not only involving the OC but participation by OB and various cellular molecules, including over 60 proteins involved in OC regulation pathways. It is important to understand the biology behind the process in order to be able to treat bone disease and reduce associated morbidity.

2.1.1 Osteoclast differentiation and activation

OC are derived from hematopoietic stem cells through the monocyte/macrophage lineage. Monocyte/macrophage progenitor[1] cells commit down the monocyte lineage where they differentiate into mononuclear OC. Mononuclear OC then fuse together to from the characteristic multinuclear OC [23]. OC differentiation requires the presence of two essential membrane bound proteins; macrophage colony-stimulating factor (M-CSF) and receptor activator for activation of nuclear factor κβ-ligand (RANKL), produced by neighbouring OB and bone marrow stromal cells [24] [25]. M-CSF, a hematopoietic growth factor, binds to the M-CSF receptor c-Fms on pre-OCs providing the signals required for their survival [26] whereas RANKL is responsible for the differentiation, fusion, activation and survival of OC [28]. RANKL has two receptors; RANK, a membrane-bound receptor on pre-OCs and osteoprotegerin (OPG), a soluble factor produced by OB and bone marrow stromal cells [23]. Upregulation of RANK promotes osteoclastogenesis whereas

1 A **progenitor cell**, similar to a stem cell, has the tendency to differentiate into a specific type of cell, but is already more specific than a stem cell and is committed to differentiate into a particular cell lineage

upregulation of OPG inhibits osteoclastogenesis. OPG is known as a 'decoy' receptor for RANKL [27], blocking the binding of RANKL to RANK, thus the RANK/RANKL/OPG system is deemed the central regulator for OC differentiation [28].

2.1.2 Osteoclast cytoskeleton and attachment to bone

Before OC can perform their resorptive function they must first attach themselves to the bone matrix. An OC membrane protein, namely the $\alpha_v\beta_3$ integrin, binds to arginine-glycine-aspartic acid (RGD) sequences on bone matrix proteins (osteopontin and bone sialoprotein) [29] to form a seal between bone and the plasma membrane of the cell. Once attached, OC become polarised in an apico-basal direction and form the characteristic ruffled plasma membrane (ruffled border) and an actin-rich clear zone [4] (Figure 2.1). The ruffled border is made up of many filopodia (finger-like projections) ~2-3μm in length and 150nm in diameter, which have been shown to penetrate the bone matrix to a depth of ~1μm [30]. The clear zone is a specialised adhesion structure located within the cytoplasm of the OC. Within the clear zone there is an actin cytoskeleton, containing two organisational forms of F-actin; the actin ring (also known as a sealing zone) and a podosome belt. An actin ring is a continuous band of F-actin whereas a podosome belt is made up of multiple columns of F-actin [31].

The organisation of the cytoskeleton varies during the resorption cycle. Väänänen and Horton (1995), have shown the formation of three distinct organisations of F-actin as OC activity changes from non-resorbing to resorbing [32]. Leading up to resorption, podosomal F-actin is distributed at the periphery of the clear zone forming clusters of dot-like structures. Closer to the main resorptive event the podosomes coalesce at the 'chosen' resorption site. When resorption begins the dot-

like podosomal arrangement is lost and a continuous actin ring predominates [32]. [11][33].

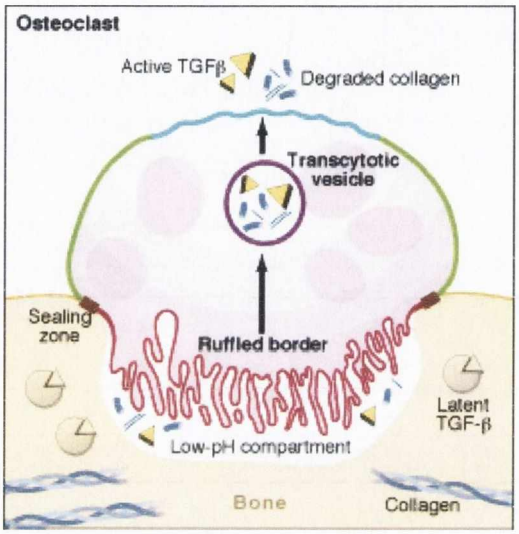

Figure 2.1: Characteristic features of an osteoclast (modified from [34]). The apex (blue), basolateral membrane (green), ruffled border (red), actin-rich clear zone (brown) and transcytotic vesicles (purple) are all characteristic features of the OC.

The unique function of both the podosome and the actin ring is highlighted in [31]. Podosome belts formed only on spread OC adhered to non-mineralised substrates whereas actin ring bands formed only when OC became polarised once adhered to mineralised substrates [31]. Their results suggested that; 1) OC have an alternating stationary resorptive phase with the presence of a sealing zone and a non-resorptive migratory phase in the absence of any specific actin structure, 2) the presence of apatite substrate promotes actin ring formation and 3) podosomes do not fuse together to form an actin ring [31]. Although the actin ring consists of structural units related to individual podosomes it is not the same as a podosome belt [35]. An

actin ring is a dense band of highly interconnected actin filaments of ~4μm diameter whereas a podosome belt consists of dense areas of actin filaments ~1μm diameter but these areas are less interconnected [36].

2.1.3 Osteoclast resorption

Resorption of bone matrix is associated with OC polarisation and actin ring formation prior to acid efflux. Once polarised the OC forms a highly convoluted ruffled border which in turn forms an isolated extracellular compartment, where net acid-transport and subsequent bone matrix degradation occur [27]. Osteoclasts resorb bone via two degradative mechanisms; 1) secretion of acid to dissolve the inorganic phase and 2) secretion of proteolytic enzymes to break down the organic matrix [37], with dissolution of the inorganic phase preceding bone matrix degradation [27].

2.1.3.1 Acid secretion

In 1816, John Howship (Howship's Lacunae) described the importance of a cellular component for the removal of bone and he indentified roughened bone regions as being resorbed [38]. In 1951, Cretin used histochemical staining to show that the osteoclastic resorption site was acidic in nature and that osteoblast attachment regions were alkaline [38]. By the 1980s, with these findings and other physiological and histochemical evidence [39][40][41][42], net acid transport was recognised as an integral mechanism of bone catabolism [38]. Two models were hypothesised for the acid-bone degradation mechanism; the first hypothesised that citrate (weak acid) solubilised bone mineral via calcium (Ca^{2+}) chelation (removal of Ca^{2+}) at bone

resorption sites whereas the second proposed a transmembrane proton transport model [38]. The second model displaced the first based on experimental evidence and is now the accepted model of osteoclast acid secretion (Figure 2.2) [15][43].

Acid secretion into the extracellular compartment requires vast amounts of energy in order to dissolve bone mineral and catalyse uptake of Ca and phosphate (P) into the cell. Mitochondria (biological energy stores) are in abundance within the OC and provide energy required for mass acid transport. Glucose within the mitochondria is converted to adenosine triphosphate (ATP) which is used in the proton-adenosine triphosphatase (H^+-ATPase) transmembrane pump that transports protons (H^+) across the ruffled membrane [44]. The H^+-ATPase pump only transports H^+ therefore, to balance charge, there are chloride (Cl^-) channels located within the ruffled border[45]. Successive release of Cl^- ions into the extracellular compartment forms hydrochloric acid (HCl), which dissolves the bone mineral and releases Ca^{2+} into the extracellular compartment. In order for OC to maintain a stable intracellular pH (pH ~4.5) [46] there is a passive chloride-bicarbonate ($Cl^-/HCO3^-$) exchange in the basal membrane to balance the acidic nature of the H^+-ATPase pump [47]. Carbon dioxide (CO_2), produced from the oxidation of glucose, interacts with water to produce carbonic acid (H_2CO_3) (Equation 2.1). Carbonic anhydrase II is responsible for maintaining balance of this reversible equation [48]. H^+ produced is used in the H^+-ATPase pump whereas the bicarbonate ion ($HCO3^-$) is used within the $Cl^-/HCO3^-$ exchange [45].

$CO_2 + H_2O <> H_2CO_3 <> H^+ + HCO3^-$ **Equation 2.1**

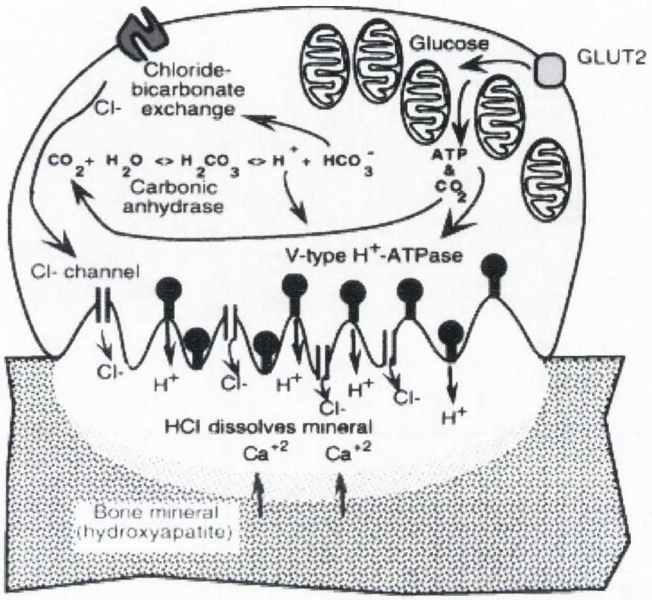

Figure 2.2: Osteoclast acid-ion-transport mechanisms [45]. Acid released into the extracellular compartment is coupled with chloride release to maintain intracellular pH.

2.1.3.2 Enzymatic secretion and endocytosis

Once acidic dissolution of the inorganic bone mineral has occurred the remaining organic component (mainly collagen) is degraded by a proteolytic enzyme, cathepsin K, finally forming the characteristic resorption lacunae ('pit') [49][18]. It has also been suggested that the metalloproteinase (MMP), collagenase, may play a role in the dissolution of organic matrix [50][51]. A collagenous gel, endocytosed[2] by the OC, is responsible for controlling secreted and resorbed products from accumulating

2 **Endocytose:** The engulfment of products from outside a cell membrane.

beneath the cell [52]. The collagenous gel acts like a filter system limiting the diffusion of products as opposed to a static impermeable barrier, previously suggested by researchers that would either allow solutes in or out. The authors hypothesised that his type of sealing zone may in fact allow the OC to both migrate and resorb at the same time [52]. This seems to contradict previously cited results where OC have an alternating stationary resorptive phase with a non-resorptive migratory phase [31][53].

After enzymatic degradation, OC then endocytose the bone degradation products (Ca^{2+} and degraded collagen) using small intracellular vesicles, which are then transported to the apical cell surface membrane and released into the extracellular space [54][55].

2.1.4 In vitro Model systems for osteoclast resorption

To date there have been many developments with respect to *in vitro* OC resorption assays [37][6][56]. Previous OC models for *in vitro* testing involved; the isolation of OC from animal bone [39][40][42][57][58], animal [59,60] and human bone marrow [61][62] and giant cell tumour tissue [63][64] or by the differentiation of mononuclear cells from human peripheral blood [61][65][43].

Primary OC are mature OC isolated from bone and do not require differentiation whereas differentiated OC are derived from pre-OCs, present within the bone marrow or peripheral blood, that require differentiation through the addition of M-CSF and RANKL to the culture medium or by co-culturing with OB (which provide M-CSF and RANKL).

Primary osteoclast cultures

Using feline bone marrow cells, Testa et. al (1981) were among the first researchers able to generate OC *in vitro*. Not only did they generate OC but they were able to maintain cultures for up to 18 weeks. Multinucleated cells with diameter ~300μm were present from 1-2 weeks [59]. OC isolated from both neonatal [58] and laying chickens [39] have been shown to survive in culture for 10 days and three weeks respectively. These studies all demonstrated that OC were able to be maintained in culture without losing their characteristic morphological traits such as; the presence of a ruffled border and a multinucleated cytoplasm with large numbers of mitochondria.

Using OC derived from neonatal rabbit bones, Chambers et. al (1984) were the first to study the resorptive activity of isolated OC on human cortical bone [40]. OC were shown to resorb bone after 24h, with pit depth ranging from ~3-5μm and the surface area resorbed ranging from ~400-1400μm^2 [40]. Large variations in surface area were attributed to differing pit morphologies; circular (usually deeper, with a small surface area), elongated (shallower with a larger surface area) and irregular (complex shapes, usually shallow with a large surface area) [40]. Adopting the previous technique [40], Jones et. al (1984) reported little difference in resorptive activity between species (rabbit, rat and chicken) [66], highlighting the potential of animal models for the quantification of OC resorption *in vitro*. However, there are associated drawbacks with such systems. Obtaining OC from bone marrow is difficult as OC are embedded within the bone matrix and are hard to isolate, they are also very fragile when isolated and can be difficult to culture [56]. Furthermore, as mature OC account for ~1% of cells associated with bone and bone marrow [67] it is extremely difficult to isolate enough cells [37].

RANKL-mediated osteoclast formation

Identification of RANKL [68] has helped combat the associated problems with isolated OC cultures and allowed for the reliable production of mature OC i.e. differentiated OC from primary bone marrow or circulating precursor cells [56]. RANKL-mediated OC generation model is now routinely used to culture OC *in vitro* [56].

Development of a pre-OC cell line derived from murine macrophage cells (RAW 264.7) [69] has now made it possible to study OC resorption more readily. RAW 264.7 cells were established from the tumour of a male mouse which was induced by the injection of Abelson leukaemia virus [69] and similarly to primary cells, it uses RANKL to mediate the differentiation of pre-OC into mature OCs [70]. The RAW 264.7 cell line has proven to be a suitable model for the generation of mature OC [71][72][73][74][75][76][77] having many associated advantages, including; 1) the availability of cell-lines, 2) the homogenous nature of pre-OC to produce OC only, 3) with the use of RANKL, a large number of mature OC are generated within days [56] and 4) RAW 264.7 cells do not require the use of M-CSF to induce differentiation [72].

2.2 Properties of calcium phosphate bone substitute materials

To address the requirements of structurally impaired bone, through disease or fracture, one must consider the properties of healthy functional bone. As discussed previously bone is a dynamic tissue that has important functions beyond structural support. However, the initial integrity of a bone substitute material is strongly influenced by its ability to function under mechanical load. Table 2.1 highlights how

structural variation within bone leads to considerable variation in mechanical properties [5]. Orthopaedic bone substitutes must therefore meet the mechanical requirements of bone, which vary with anatomical location.

Table 2.1: Mechanical properties of bone and common calcium phosphate ceramics.

Material	Compressive strength (MPa)	Tensile strength (MPa)	Young's modulus (MPa)	Reference
Cortical bone	130-193	50-150	12-18 x 10^3	[5][17]
Cancellous bone	3-12	-	100-500	[5][17]
TCP (dense)	120	-	-	[78]
HA (0.1-3% porosity)	350-450	38-48	7-13	[78]

The properties of CaP ceramics differ significantly with composition, crystallinity, grain size and porosity [17]. However CaPs, regardless of form (powder, bulk, porous or coating) or phase (amorphous or crystalline) have been shown to support the attachment, differentiation and proliferation of bone cells *in vitro* [64][79][80] and *in vivo*[17][81][82][83]. It is this inherent biocompatibility of CaPs that make them a material of choice for bone substitute materials.

The application of a particular CaP is determined by the overall degradation rate of the material which is in turn determined by the same factors used to characterise the material; chemical composition, microstructure and solubility. The two most widely used phases of CaP ceramics are HA and β-TCP which have Ca/P ratio of 1.67 and 1.5 respectively [78]. In bone the ratio of calcium to phosphorus (Ca/P ratio) is ~1.67 [78]. For synthetic CaPs a lower Ca/P ratio produces a more acidic material that solubilises more readily in water and thus degrades faster *in vivo* however materials with a Ca/P ratio less than 1 are considered unsuitable due to their high

solubility and acidity [84]. At physiological pH (pH 7.0) degradation of synthetic CaPs is in the order; monocalcium phosphate (MCPM) > α-TCP > Dicalcium phosphate dihydrate (DCPD) > Dicalcium Phosphate (DCP) > Octocalcium phosphate (OCP) > β-TCP > HA, with HA exhibiting the slowest degradation [22] [17]. Table 2.2 summarises the main biologically relevant CaP materials and their respective Ca/P ratios.

Table 2.2: Calcium phosphate compounds [22][21].

Name	Abbreviation	Formula	Ca/P
Dicalcium phosphate (monetite)	DCP	$CaHPO_4$	1.00
Dicalcium phosphate dihydrate (brushite)	DCPD	$CaHPO_4.2H_2O$	1.00
Octacalcium phosphate	OCP	$Ca_8(PO_4)_4(HPO_4)_2.5H_20$	1.33
Tricalcium phosphate	TCP	$Ca_3(PO_4)_2$	1.5
Hydroxyapatite	HA	$Ca_{10}(PO_4)_6.(OH)_2$	1.67

Appropriate material selection can also be made on the basis of material degradation rate relative to the site of implantation. Head and mandible resorption occurs at a faster rate than most other skeletal bones [85][86] with craniomaxillofacial applications requiring relatively fast degrading materials compared to spinal applications which require slower degrading materials that retain their mechanical integrity.

Resorbable bone substitutes for biomedical applications should have a porous structure to optimise bone-biomaterial integration. Introducing porosity into a bone substitute increases the surface area available to bond with the host bone and allows infiltration of blood and cells into the implant site. Porous bone substitute materials

are termed scaffolds, with a structure strong enough to support and protect the local environment but one that permits entry to stimuli required to modify the existing structure. Microporosity (pore diameter <10μm) determines the resorbability of the porous scaffold [81] and is defined by the impregnation of the scaffold by biological fluids [87] whereas macroporosity (pore diameter >100μm) determines the degree of integration of the scaffold with the host bone [19]. The 'optimal' macroporosity for bone ingrowth is in the range of 100-600μm [88][89][90]. But these figures are controversial as experimental results for the determination of an optimum macropore size differ widely and are often contradictory for both implant resorption and bone ingrowth [83][88] [91] [92]. Macroporosity has been given priority when trying to improve CaP scaffold osteointegration *in vivo* however recent research has indicated that the presence of microporosity may have the ability to improve osteointegration. Osteocytes can embed within the micropores and have the potential to form a mechanosensory network within the scaffold. This network may improve mechanical properties of the scaffold and overall scaffold performance [90][93][94].

2.3 Resorption activity on calcium phosphate bone substitute materials

When designing a CaP implant for bone repair, material selection should be chosen on the basis of degradation or remodelling rate; for example, β-TCP has a fast degradation and HA is slowly remodelled [95]. Other determining factors when considering the suitability of a material as a bone substitute material are the size and location of the defect [95]. Studying the mechanism by which OC degrade CaP ceramics can also provide insight into the suitability of various candidate ceramics for use as bone substitute materials. Comparisons made between individual ceramics on the extent and rate of degradation by OC provides important data for the design or

modification of bone substitute materials however, data obtained from *in vitro* studies has often been contradictory; with some authors observing OC resorption of HA [64] [95] and others not [96].

The traditional *in vitro* method used to assess resorbability of bone substitutes is a cell-based resorption assay, alternatively known as a 'pit' assay, developed by Boyde [57] and Chambers [40] in 1984. The initial assay was developed using dentine and bone respectively [57] [40], but is now routinely used to understand biomaterial resorption. OCs are cultured on the bone substitute surfaces for specific periods and are then detached, at which point the excavated areas (pits) beneath the cells can be analysed by scanning electron microscopy (SEM). This has led to elucidation of several material properties that can affect OC-mediated resorption of ceramic surfaces.

2.3.1 Factors affecting resorption

2.3.1.1 Ceramic material parameters

Composition and solubility

Material composition and solubility are inter-related with respect to CaP degradation dynamics. Even small differences in the composition of CaP bone substitutes can have a large effect *in vivo* [22] with the subsequent changes in phase composition directly affecting the cell-ceramic ionic exchange dynamics [21][96][97].

A BCP with HA to β-TCP ratio 60/40 has been used for clinical application; a compromise between solubility and mechanical strength [98]. Yamada et al. (1997) used OCs isolated from neonatal rabbit bone to study the effect of different HA/β-

TCP ratios on the resorptive behaviour of OC [96]. The materials studied were pure β-TCP, pure HA and two types of BCP with HA to β-TCP ratios of 25/75 and 75/25 respectively. After 2 days in culture there was no apparent difference in size or appearance of the cells on either material however there were noticeable differences in resorptive activity. On pure β-TCP resorption was intermittent; on BCP 25/75 resorption was more continuous than that of pure β-TCP. Both BCP 75/25 and pure HA showed no resorption [96]. These results suggested that OC resorbed the materials differently depending on phase composition. Although there is some correlation of resorption with solubility of the ceramics the trend observed was not linear with increasing solubility. BCP 25/75 was the most extensively resorbed material yet it is less soluble in acidic solution than pure β-TCP. Yamada et al. (1997) concluded that OC preferred the BCP surface, perhaps allowing the OC to resorb in a manner similar to their behaviour on natural bone [96].

Similarly, Monchau et al. (2002) conducted an *in vitro* study investigating the resorptive activity of OC on HA, β-TCP and calcium carbonate ceramics [64]. On HA the excavated areas were extended and reached an average diameter of ~86μm, with β-TCP the excavated areas were deeper but had a slightly smaller average diameter of ~61μm however the lacunae were numerous and had well-defined boundaries. For calcite, the degraded areas were strongly extended and very superficial with an average diameter of ~97μm [64]. OC were isolated from a human giant cell tumour and a new-born rat; the degraded areas showed comparable resorptive behaviour for rat and human OC on all three materials. This study highlights how less soluble materials such as HA undergo superficial attack from OC whereas the more soluble β-TCP is resorbed to a greater extent with continuous resorption trails of significant depth [64].

These studies show that OC can resorb CaP ceramics but that the extent of resorption is dependent upon the composition and inherent solubility of the ceramic. Generally, a more soluble material displays a higher resorptive capacity to a less soluble material. However pure β-TCP, which is more soluble than a BCP of β-TCP and HA, may be too soluble for OC activity and result in a reduction in resorption. Altering the CaP ratio may therefore provide a simple but effective way to control OC resorption and subsequent bone formation *in vivo.*

Presence of metal ions

Bone mineral contains many trace elements all of which play an important role in the biological processes associated with bone growth and turnover [21][99]. It has been shown that addition of Magnesium (Mg^{2+}) [78], strontium (Sr^{2+}) [73][99], copper (Cu^{2+}), zinc (Zn^{2+}), fluoride (F^-), carbonate (CO_3^{2-}) [99] silicon (Si) [100][101] and cobalt (Co^{2+}) [102] ions to CaP has a significant effect on OC resorption *in vitro*. Based on an increase in ion concentration the effects on OC resorption *in vitro* are either; negative/inhibitory (Sr^{2+} [73], Cu^{2+}, Zn^{2+}, F^-, CO_3^{2-} [99]) or positive/stimulatory (Mg^{2+} [78], Si [100], Co^{2+} [102],). The main routes for ion incorporation into CaP ceramics is either ion substitution (CaP contains the ions) or ion coating (CaP surface is coated with ions).

Strontium Ranelate (trade name, Protelos), a combination of strontium and ranelic acid, is the current drug used to treat osteoporosis [103]. Strontium Ranelate stimulates OB activity whilst inhibiting OC activity, thus promoting net-bone formation. Based on this finding, a Sr^{2+} -substituted bioglass (BG) (0%, 10%, 50% and 100% Ca^{2+} content substituted by Sr^{2+}) was produced to develop a bone substitute material that could both promote bone formation and reduce bone resorption [73]. RAW 264.7 cells were cultured for 6 days on Biocoat Osteologic

CaP coated slides with a conditioned culture medium containing dissolution ions from the Sr^{2+}-substituted BGs. Cultures that received dissolution ions from 10%, 50% or 100% Sr^{2+}-substituted BG displayed significantly less TRAP activity compared to control cultures (0% Sr^{2+}-substitution) and resorption pit area decreased with increasing Sr^{2+}-substitution [73]. The mechanism by which Sr^{2+} inhibits OC resorption has not been defined but it may be that Sr^{2+} inhibits OC differentiation, disrupts cytoskeletal stability or prevents the resorptive activity of mature OC [73]. OC generated from the Saos-2, human osteosarcoma cell line, were cultured on tissue culture plastic in the presence of dissolution ions from Sr^{2+}-substituted BG. OC displayed flattened non-resorbing morphologies.When Saos 2 OC were placed on the Sr^{2+}-substituted BG typical OC apico-basal polarisation of the cell was observed. This suggests that OC polarisation is substrate specific [31].

Primary rabbit OC were used to investigate the effects of Sr^{2+} , Cu^{2+}, Zn^{2+}, F^- and CO_3^{2-} incorporation into CaP on resorption *in vitro* [99]. The ions were incorporated into CaP films and cultured for 40 h. There was a significant decrease in the number of resorption pits formed for all additive films as compared to control films without additives [99]. The inhibitory effect was concentration dependent, with increasing additive concentration decreasing resorption pit formation [99]. The addition of trace elements did affect resorptive activity but did not affect the attachment of cells as no significant difference was found for the total number of OC between groups [73][99]. The effects may have been a result of either: 1) the incorporated additives present within or on the surface of the film may have directly affected cell response and 2) additive incorporation may have changed the physiochemical properties of the CaP film, having an indirect effect on cell response [99].

Primary rat OC were cultured *in vitro,* on Si^{2+}-substituted microporous HA (Si-mHA) thin films sintered between 800-1000°C. Pellets of Si-mHA, and BCPs

(HA:β-TCP) were also tested [100]. A cell-free *in vitro* dissolution experiment (measuring Ca^{2+} release) was also conducted on Si-mHA, mHA, β-TCP, α-TCP and commercial HA (cHA) pellets. An increase in % Si-TCP content increased resorption for Si-mHA films. Only samples sintered at 1000°C showed significant levels of resorption indicating that surface morphology influenced resorption. Si-mHA pellets exhibited resorption whereas mHA and BCP pellets (predominately β-TCP) did not. The dissolution tests also showed β-TCP pellets to have the least Ca^{2+} release as compared to Si-mHA, mHA, cHA and α-TCP pellets [100]. Based on the solubility of HA and β-TCP the opposite effect should have been observed with β-TCP dissolution and subsequent resorption preceding that of apatite containing compounds [104] . Perhaps the apatite samples may not have been highly crystalline, containing an amorphous phase – therefore increasing the solubility of the apatite samples [104].

A bulk Si-mHA was implanted into male rats *in vivo* [100]. *In vivo* results showed evidence of remodelling of the Si-mHA implant after 6 weeks with migration of new bone tissue into the microporous structure of the implant and the presence of active OC at the bone-implant interface [100]. As there are no quantitative results for Si^{2+} release *in vivo* it cannot be assumed that the presence of Si^{2+} within the implant influenced OC recruitment and activity *in vivo* nor is there a control material to compare *in vivo* OC response. TRAP staining was carried out on *in vitro* cultures [100] however OC number is not reported therefore it cannot be determined if material composition or microstructure affected the initial attachment of the OC and subsequent resorptive profiles.

The benefits of incorporation of metal ions into CaP and their mechanism of action is controversial. Is it due to: a) the direct incorporation of the ions into the CaP, b) the indirect incorporation of ions into the CaP (changing physiochemical material

parameters such as density, solubility, grain size, pore size) or c) a combination of both factors? [104]. Some suggest that Si^{2+}-substituted CaPs have a "superior biological performance to stoichiometric counterparts" [101]. But others point to the fact that there is no experimental evidence that Si ions are released from Si-substituted calcium phosphates at therapeutic concentrations and remain sceptical of the benefits of ion substitution [104].

In more recent studies, an attempt has been made to quantify the release of Co^{2+} ions in culture medium from a Co^{2+}-substituted CaP [102]. Murine bone marrow OC were cultured on CaP coated tissue culture plates containing a range of Co^{2+} concentrations (0.1-20μm). An increase in OC differentiation and activity relative to Co^{2+} free control groups was observed. Initial formation of OC was influenced by lower levels (0.1-1μm) of Co^{2+} incorporation, OC activity levels peaked within a range of 5-20μm and resorption occurred between 0.1-5μm [102].

The cell culture used in this study consisted of primary cells, presumably of differing stages of maturation. This heterogeneity has been proposed as a plausible reason for: 1) OC resorption occurring over a wider range of concentrations compared to the lower concentration that favoured OC formation and 2) why OC activity did not correlate with OC formation across the various concentrations [102] i.e. cells at different stages of maturation will display different activities. Regardless of the lack of correlation between OC formation, activity and resorption OC directly responded to Co^{2+}.

Several review articles have extensively reported the biological response to ion-substituted CaPs [78][105][106][107]. The mechanism as to how the addition of trace elements affects the cellular response requires much more attention. Despite the controversy, ion-substituted materials could provide suitable modification for the

control of bone formation and resorption *in vivo* however an improved *in vitro* assay is required to understand more clearly how they are resorbed..

2.3.1.2 Culture media variants

Addition of metal ions

Similar to incorporation/coating of ions to CaP materials, OC resorptive activity can be inhibited or stimulated by the addition of ions to culture medium. An investigation on the effects of a Zn^{2+} supplemented TCP ceramic against a zinc containing TCP (ZnTCP) was conducted *in vitro* [108]. Isolated OC from rabbit bones were cultured on the substrates for 6 and 24 hr [108]. An increase in Zn^{2+} (0.3, 1.3 and 6.8ppm) to culture medium had a significant increase in the ratio of apoptosis at 6 and 24 hr with a subsequent decrease in actin ring formation (denoting a reduction in OC resorptive activity). ZnTCP, formulated to release comparable Zn^{2+} levels inside the resorption lacunae, did not influence OC apoptosis or actin ring formation [108]. The presence of Zn^{2+} only had an effect on OC activity when ions were added to the surrounding media. No effect was observed when Zn^{2+} was inside the resorption lacunae [108]. These effects are explained by the presence of a plasma membrane zinc transporter (ZIP1). High Zn^{2+} concentrations outside the resorption lacunae (in the surrounding medium) can be taken up by ZIP1 on the basal membrane of the cell and also at the sealing zone subsequently inhibiting OC activity and inducing apoptosis. This is in contrast to high Zn^{2+} concentrations within the resorption lacunae, here they cannot be taken up by ZIP1 and permeate out of the OC without affecting resorptive activity . However OC activity was measured by the formation of actin rings, and in the author's experience actin ring formation is indicative of OC resorptive activity but does not correlate with resorption. Therefore it would have been preferable to have included resorption pit formation as an

outcome measure in order to be able to correctly gauge the extent of OC resorptive activity on addition of increasing Zn^{2+} concentration within the culture medium.

In contrast and as previously discussed, Co^{2+} addition to culture medium was shown to increase resorptive activity [102]. Therefore, OC response to metal-ion addition to culture medium appears to be element specific. This may be related to the specificity of OC membrane ion channels to particular ions or indeed the selectivity of OC to the uptake of released substrate products within the extracellular compartment. Experiments of this type provide evidence of the effects of individual ions at a range of concentrations however it is difficult to relate this to the *in vivo* environment where the local concentration of elements can change with systemic changes and thus is not a static situation.

Extracellular calcium concentrations

An increase in extracellular calcium (ECa^{2+}) has been shown to reduce OC podosome formation [109] and increase intracellular calcium ($Ca^{2+}{}_i$) [110]. Both effects reduce the resorptive capacity of the OC. It has also been observed that an increase in H^+ concentration reduces $Ca^{2+}{}_i$ and subsequently increases OC resorption [111]. These findings suggest that cytosolic Ca^{2+} acts as a second messenger, indirectly controlling OC resorption. When OC attach to bone, the sealing zone forms an isolated micro-environment where bone resorption takes place. As the bone mineral dissolves, the Ca^{2+} concentration within the microenvironment can increase from 8- 40 mmol/L (320 – 1600 mg/L) [46]. This local increase in Ca^{2+} concentration increases $Ca^{2+}{}_i$ promoting margin retraction and OC cell dehesion [112] from bone, ceasing resorption. Once the OC detaches from bone the cytoplasm is continuous with the surrounding extracellular environment where the accumulated by-products of resorption can then be released. This sequence of events highlights

how an increase in ECa^{2+}, produced during active resorption, creates a negative feedback loop to prevent further resorption (See Figure 2.3).

Inevitably with CaP materials, ECa^{2+} values will vary relative to ceramic solubility. *In vitro* studies show that the higher surface dissolution of TCP ceramics compared to HA cause an increased Ca^{2+} concentration within culture media, inhibiting OC migration [95].

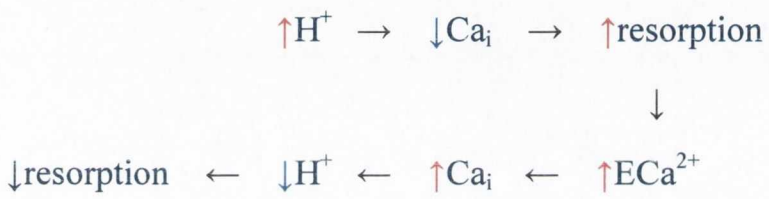

Figure 2.3: A simplified schematic diagram outlining the H^+/Ca^{2+}/resorption negative feedback loop. ECa^{2+} created by the resorption process inhibits further resorption.

Therefore highly soluble CaP ceramics could be unsuitable for natural OC resorptive activity as a high Ca^{2+} concentration generated within the localised compartment of the attachment site inhibits natural resorptive behaviour [64][95][96]. Non-active OC detach from bone and go through a migratory phase. Intermittent surface resorption on highly soluble β-TCP has been reported [96]; the cells actively resorbed for a short time before being inhibited where they then detached and migrated before resuming resorptive activity. These findings show the inter-relationship between material composition, solubility and OC resorption. The solubility of a particular CaP determines OC activity and subsequent material resorption hence; solubility may prove to be a critical determining factor when

designing a resorbable CaP bone substitute material. A particular CaP ceramic may have a more viable solubility profile for OC resorption compared to another.

There have been many suggestions for the mechanism of calcium regulation within and surrounding the OC microenvironment. Voltage-gated calcium channels located within the OC plasma membrane have been shown to regulate resorption; an increase in ECa^{2+} from 2-4mM reduced resorption by 50% [113]. Calcium-Sensing Receptors (CaSR) on the surface of OC have also been shown to regulate OC resorptive activity through interaction with ECa^{2+} [114]. One of the most debated issues is the route of disposal of Ca^{2+} from the resorptive site. The main mechanisms described are; 1) bulk transcytosis [115], involving Ca^{2+} release from OC via vesicular transport and 2) continuous disposal [116], involving intracellular Ca^{2+} channels and membrane pumps [117].

OC mitochondria may also be a carrier of Ca^{2+} within the OC. In general, the concentration of Ca^{2+} within the cell cytoplasm is $\sim 10^{-7}$ M and markedly higher in the mitochondrial matrix at $\sim 10^{-3}$M [118]. An *in vitro* study examined the relationship between Ca^{2+} accumulation in OC mitochondria and bone resorption. Murine bone marrow cells were used to generate OC *in vitro* and were cultured on dentine for 24h. Electron Energy Loss Spectroscopy (EELS) was used to examine the localisation of Ca^{2+} within OC mitochondria [118]. It was observed that OC mitochondria close to the ruffled border of the attachment zone were rich in mitochondrial granules and contained a high Ca^{2+} concentration whereas mitochondria in the basolateral region contained few granules [118]. The identification of high concentrations of Ca^{2+} within mitochondrial granules close to the site of resorption implies that there is a relationship between OC mitochondria and Ca^{2+} transport and release during resorption of bone matrix [118]. It is believed

that Ca^{2+} accumulation in mitochondrial granules helps prevent a build up of Ca^{2+} in the cytoplasm of resorbing OC [118], preventing inhibition of resorptive activity [64][95]. This may help to explain how OC can still perform their resorptive function, up to a critical point, in relatively high Ca^{2+} environments in the presence of degrading CaP materials.

pH of culture medium

Osteoclasts are known to be pH sensitive and have an intracellular pH of ~4.5 [45]. As previously discussed, protons (H^+) are transported across the ruffled membrane into the extracellular compartment to degrade bone mineral [45]. An *in vitro* experiment using rat osteoclasts tested the stimulatory effect of protons on osteoclast resorption [119]. The study revealed a positive, dose-dependent effect of extracellular protons on resorption of bone slices, a 14-fold increase in resorption was observed with a change in pH from 7.4-6.8 [119]. The experiment was carried out in the absence of bicarbonate and carbon dioxide, normally found in culture medium, to ensure the main independent variable was the hydrogen ion concentration.

Further emphasis on the pH sensitivity of osteoclasts is illustrated by Arnett and Spowage (1996) who used a HCO_3^-/CO_2 buffered medium, to simulate the physiological buffering system found within osteoclasts [120]. Results from the rat osteoclast study showed that the sensitivity of rat osteoclasts to pH was within a more limited range. At pH 7.3 or above, little or no bone resorption was observed but at pH 7.0 osteoclasts exhibited near maximal resorptive activity. Between pH 7.15 -7.25 there was a 6-fold increase in the number of resorption pits formed [120]. Thus, as little as 0.1 unit of pH produced a large difference in resorptive activity,

demonstrating the sensitivity of osteoclasts to changes in pH (analogous to an "on/off switch") [120].

2.3.2 Resorption or Phagocytosis?

In vivo degradation of CaP ceramics occurs through a combination of cell-mediated OC resorption and passive dissolution. With regards to cell-mediated degradation, there has been speculation about the resorptive capacity of OC and the relation to phagocytosis. Many believe that OC primarily degrade ceramic surfaces through resorption [97] but others feel that phagocytosis may have a larger role to play than previously thought [personal communication with Prof. Willy Hofstetter (Osteoclast biologist, Bern University, Switzerland)]. Since OC are derived from a monocytic macrophage lineage it is not unreasonable to think that OC may degrade ceramic surfaces through phagocytic mechanisms and that other macrophage cells may contribute to ceramic degradation by phagocytosis. The debate that presently stands is if OC do phagocytose the ceramic particles does this eliminate the role of resorption as an OC degradation mechanism? [97]. Or is ceramic degradation a result of a combined cellular effect, with OC primarily degrading by resorption and macrophage cells degrading by phagocytosis? Or can OC simultaneously resorb and phagocytose ceramic surfaces?

An *in vitro* degradation study of a BCP, (40:60 weight % of HA and β-TCP respectively) using OCs derived from neonatal rabbit bone cells showed degradation to occur via simultaneous resorption and phagocytosis mechanisms [121]. Whilst resorbing the surface of the CaP the OC cell cytoplasm starts to close around groups of degraded CaP particles eventually closing to form a vesicle containing the degraded CaP particles. Once inside the OC, the vesicle plasma membrane disappears releasing the phagocytosed crystals into the cell cytoplasm. CaP particles

were then shown to undergo *in situ* fragmentation and dissolution within the cell [121]. A 6 week *in vivo* study of HA degradation by OC in sheep also showed OC-mediated degradation of CaP ceramics to occur by simultaneous resorption and phagocytosis [97]. Similar to Heymann et al. (2001), Wenisch et al. (2003) reported OC to simultaneously phagocytose the resorbed CaP particles into large intracellular vesicles; through large extensions of the cytoplasmic membrane [97]. However, Wenisch et al. (2003) observed that particle phagocytosis could also occur when the ruffled border formed deep invaginations into the cell cytoplasm, a mechanism not described by Heymann et al (2001) [97], but one that ultimately formed intracellular vesicles containing degraded CaP particles followed by subsequent particle fragmentation.

In addition to phagocytosing inorganic ceramic material, OC have been shown to phagocytose both polymeric and metallic biomaterial particles of various sizes [63]. Human OC from giant cell tumours of bone and rat OC from long bones were cultured on cortical bone slices in the presence of latex, polymethylmethacrylate (PMMA) and titanium. After 3days, both rat and human OC exhibited resorption of the cortical bone slices and phagocytosis of biomaterial particles in all cultures, whilst maintaining OC phenotype [122].

The effects observed by these studies are not surprising and are in-keeping with the acidic nature of the OC and the vacuolar transport system that allows bone degradation products to be packaged into small intracellular vesicles and transported to the cell surface membrane to be released [45]. However, these studies have confirmed that phagocytosis does not abolish the resorptive activity of the OC and that both degradation mechanisms can occur simultaneously without negative effect allowing the OC to remain fully functional.

In summary of the literature above there are many factors to consider when designing a resorbable CaP bone substitute material. These include mechanical properties, material composition and solubility, osteoconductivity and rate of resorption. For instance, a highly soluble material would inhibit material resorption and have poor mechanical properties. A BCP composite can be used to counteract this by incorporating a less soluble material with a more soluble material to retain strength but have a moderate solubility profile and therefore better resorption. There is also the option to coat or incorporate metal ions into CaP bone substitute materials to promote cell attachment and proliferation. Other factors affecting resorption will be discussed later in the thesis and will include the effects of surrounding pH and material surface roughness.

2.4 Conclusion and current situation

Variations in cell culture protocols also contribute complexity to the problem. There is no standard routine assay used within OC cell culture labs to assess resorption i.e. there are variations in the type of media and additives used, the length of time particular OC cell types are cultured on a given biomaterial, the source and stage of OC maturation and subsequent population heterogeneity. Besides variation in OC assay parameters, the outcome measures used to assess resorption vary between studies and often do not provide an adequate representation of resorption. Resorption pit area measurements are most commonly used however; pit number and pit volume have also been reported. Other outcome measures that assess resorption are based on biochemical techniques which assess resorption as a function of OC activity. Therefore direct comparisons of different materials between studies is difficult. These variables and the challenges in quantifying their influence on resorption have formed the basis of this research.

There is an international standard (ISO) for ceramic dissolution under various conditions (DIN EN ISO 10993-14) but none for cell-mediated resorption *in vitro*. Zhang et al (2012), has proposed that, rather than establishing a standard resorption assay it may be more realistic to define a standard material to which all other materials are compared [6]. Even bovine bone has inter-donor variability therefore it would not be possible to have a standard material free of variation.

In the absence of any official standard method or material for assessing resorption the current *in vitro* method used to assess resorbability of bone substitutes is the OC pit assay. This assay assesses pit formation by quantifying either pit number, pit area, pit volume or a combination of the three. These methods are very time-consuming, often require specialised and expensive equipment and are unsuitable for use on porous scaffolds. Thus there is a need to develop an outcome measure of resorption that can be used throughout a wide range of *in vitro* experiments and one that is accessible to basic laboratories, not requiring expensive and specialised equipment but more importantly, within the rapidly developing field of tissue engineering; the ability to assess resorption of porous scaffolds. As pit area measurements seem to be the preferred method of choice the premise of this research was to utilise pit area measurements to determine the suitability of alternative measures of OC resorption *in vitro*.

2.5 Research opportunity & Thesis aims and objectives

In order to carry out this body of research a collaboration was formed between the Robert Mathy's Foundation, Switzerland and Queen's University, Belfast. Additional funding was supplied by the Department of Education and Learning. The collaboration was set up with the intent of finding a novel way of measuring osteoclast resorption *in vitro*.

Many of the methods currently used to assess the resorbability of CaP biomaterials are suitable as indicators of OC resorption on the basis of biomaterial biocompatibility, identifying possible mechanisms that modulate the rate of resorption or for the investigation of drug-OC interactions and their effect on resorption. However, there has been no systematic attempt to identify an outcome measure of OC resorption that directly correlates with pit measurements and is transferrable through a broad range of *in vitro* experiments. Although pit area measurements have become common practice when assessing biomaterial resorption *in vitro* it is not a precise indictor of resorption;

- variations in pit depth are not taken into consideration
- it is unsuitable for use on porous materials
- it is unsuitable for use on materials with rough surfaces

With that, the aim of this research was to improve on the fundamental understanding of OC resorption of CaP biomaterials by identifying suitable measures of OC resorption that directly correlate with pit measurements and are transferrable through a broad range of *in vitro* experiments.

To achieve this aim a number of objectives were set out;

- Develop an OC assay for the generation of functionally active OC for the resorption of CaP materials *in vitro*
- Assess various outcome measures as indicators of resorption *in vitro*
- Correlate these outcome measures with pit area measurements
- Validate the OC assay and respective outcome measures *in vitro* on CaP materials to assess the suitability of outcome measures for use in a broad range of *in vitro* experiments.

2.6 Plan of Experiments

The plan of experiments below serves as a reference guide to the reader for the sequencing of the experimental work of this document.

Chapter 3: Development of an osteoclast assay for the generation of RAW 264.7 osteoclast cells in *vitro*

Initial OC assay development based on the protocol by Osdoby et. al in [56].

Chapter 4: A comparison of osteoclast differentiation and function using osteoclast precursor cells and RAW 264.7 cell line

Experiment A: Investigation of the initial cell culture conditions with a primary focus on RANKL dose and cell culture period.

Experiment B: Utilisation of the cell culture conditions in Experiment A for the examination of the *in vitro* OC response to dense, polished CaP surfaces before and after calcination.

Chapter 5: Modifications to the development of an osteoclast assay for the generation and function of RAW 264.7 osteoclast cells *in vitro*

Experiment C: Determination of the effect of RANKL dose and supplier on the generation and activity of mature OC.

Experiment D: Determination of the effects of HCl addition to culture medium on β-TCP surfaces in a cell-free environment.

Experiment E: Determination of the effect of HCl addition to culture medium on culture medium pH during incubation.

Chapter 6: Surrogate outcome measures of in vitro osteoclast resorption of calcium phosphate ceramics

Determination of the suitability of several outcome measures as possible indicators of osteoclastic resorption *in vitro*.

CHAPTER THREE

Development of an osteoclast assay for the generation of RAW 264.7 cells *in vitro*

3 Development of an osteoclast assay for the generation of RAW 264.7 osteoclast cells in *vitro*

3.1 Introduction

The aim of the work presented in this chapter was to establish an experimental methodology to generate OC cells *in vitro*. A resorption assay using Murine RAW 264.7 macrophage cells was developed based on the protocol by Osdoby et al. in [56] whereby addition of RANKL to culture medium induces RAW 264.7 monocyte cells to form multinucleated OC. A cell line was chosen over human derived cells due to the inherent inter-donor variability and the difficulty in isolating and culturing these cells.

This chapter investigates the effect of three cell seeding densities on OC formation when cultured on HA and β-TCP ceramic discs sintered at either 1100°C or 1250°C (Table 3.1). OC were characterised by their expression of TRAP. Several outcome measures were investigated to assess their potential as indicators of OC resorption *in vitro*; gravimetric analysis, pit formation viewed by SEM, mineral release using inductively coupled plasma mass spectrometry (ICP-MS) and a colorimetric assay measuring TRAP5b enzyme activity in the culture medium.

3.2 Materials and Methods

3.2.1 Preparation of ceramic discs

Highly crystalline HA, $Ca_{10}(PO_4)_6(OH)_2$ (Batch no: P288R) and amorphous β-TCP $Ca_3(PO_4)_2$ (Batch no: P303R), 100% unsintered powders (both Plasma Biotal Ltd, UK) were used to produce CaP discs. Discs of HA and β-TCP were produced by die compaction using a hydraulic bench press (Sealey, YK10B). Using a cylindrical

stainless steel die (inner diameter 15mm), 0.5g of powder was pressed under 1.5kg/cm^2 of pressure to produce a single circular disc (15mm diameter x 2mm thick). HA and β-TCP discs were sintered at either 1100°C or 1250°C in an ELITE furnace. Discs were left unpolished as a flattened surface was achieved after compression. Discs were sterilised by autoclaving at 121°C for 30 minutes.

Table 3.1: Sintering profiles for HA and β-TCP discs

Temperature	Material	Sintering Programme
1100°C	HA β-TCP	Ramp rate 4°C/min, dwell time of 1h at 1100°C, cooling rate of 4°C/min to room temperature.
1250°C	HA β-TCP	Ramp rate 4°C/min, dwell time of 1h at 1250°C followed by cooling to 900°C at 1°C /min with a dwell time of 24h at 900°C, followed by a cooling rate of 1°C /min to room temperature.

3.2.2 Cell culture of RAW 264.7 cells

RAW 264.7 cells (ATCC, UK) at passage 11 were routinely cultured under standard conditions (37°C, 5% CO_2/95% air) in Dulbecco's modified Eagles medium (D-MEM), containing 4.5g/L glucose supplemented with 10% (v/v) foetal bovine serum (FBS), 1.0mM sodium pyruvate, 4mM L-glutamine and 1% (v/v) antibiotic/antimycotic (all reagents from Invitrogen, UK). At day 0, cells were seeded onto HA and β-TCP discs at a density of either 1x10^3, 1x10^4 or 1x10^5 cells/cm^2 in 24-well plates. To initiate differentiation, RANKL (R&D systems, USA)

was filter sterilised through a 0.2μm filter and added to complete culture medium at a final concentration of 35ng/mL. Culture medium and RANKL were replaced on day 3. Cultures were maintained for 6 days. N=5 for all conditions (Table 3.2).

Table 3.2: Experimental design plan

Temperature	Material	Seeding density and sample number				Total
		Cell free	1 x 10^3	1 x 10^4	1 x 10^5	
1100°C	HA	5	5	5	5	20
	B-TCP	5	5	5	5	20
1250°C	HA	5	5	5	5	20
	B-TCP	5	5	5	5	20
						80

3.2.3 Scanning electron microscopy

SEM was used to view the ceramic surface after culture with RAW 264.7 cells and to study the morphology of the cells. Day 6 cultures on discs were washed twice with PBS and fixed in 3% (w/v) gluteraldehyde in 0.1M sodium cacodylate buffer at 4°C for 30 min. After fixation, discs were washed with 0.1M cacodylate buffer for 1 h, dehydrated via graded ethanol series (50%, 70%, 90% respectively for 30 minutes at each concentration followed by three changes in 100% ethanol) followed by drying with hexamethylsilasane (HMDS) (Sigma Aldrich, UK). The dried specimens were then sputter coated with gold using a Polaron E5150 sputter coater and viewed on a Jeol SEM at 15kV. N=5 per time point for all conditions.

3.2.4 Gravimetric analysis

Gravimetric analysis was chosen as an outcome measure to determine if a change in mass could be detected after culture with RAW 264.7 cells. The mass of each ceramic disc was recorded after sintering and prior to cell culture (Initial mass, M_o) and again after critical point drying with HMDS (mass after drying, M). Results were recorded to 4 decimal places. The percentage change in mass was calculated as in Equation 3.1.

$$\% \text{ mass loss} = \left(\frac{Mo - M}{Mo}\right) \times 100 \qquad \text{Equation 3.1}$$

3.2.5 TRAP assay

TRAP has two isoforms that circulate in the blood, 5a and 5b. TRAP 5b is derived from OC and is produced by RAW OC whereas TRAP 5a is from inflammatory macrophages. TRAP 5b activity was measured in culture medium harvested at day 6 (N=5) using MouseTRAP (Immunodiagnostic systems Ltd, UK), a TRAP 5b specific-immunoassay. The MouseTRAP assay is a solid phase enzyme-linked immunosorbent assay (ELISA) specific for the determination of OC-derived TRAP 5b. In an ELISA an enzyme is used to detect the presence of a substrate (usually an antigen) in a liquid sample. A specific enzyme-linked antibody binds to the test antigen and the complex formed is then exposed to a chromogenic substrate specific to the attached enzyme. The colour intensity of the formed complex is directly proportional to the concentration of the test antigen.

Microtitre wells were coated with 100µl of anti-MouseTRAP antibodies and incubated for 60 minutes at room temperature, shaking at ~950rpm. Well contents

were decanted and 250µl of wash buffer (40mL Tris-buffered saline containing Tween-20 diluted in 960mL distilled water) was added to all wells, decanted and repeated three times. 75µl of 0.9% NaCl followed by 25µl of test sample (culture medium containing TRAP 5b antigen) was added to wells in duplicate. 25µl of release reagent was added to all wells and incubated for 60 minutes at room temperature, shaking at ~950rpm. Well contents were decanted and 250µl of wash buffer was added to all wells, decanted and repeated three times. 100µl of substrate solution (two tables of p-nitrophenylphosphate (pNPP) in 10mL sodium acetate buffer) was added to all wells. The microplate was covered and incubated at 37°C for 2 hours before adding 25µl of stop solution (0.32M Sodium Hydroxide (NaOH). After termination of the reaction, optical absorbance was read at 405nm on a microplate reader (Genios, Tecan, Austria) and the colour intensity produced was used to quantify the amount of TRAP 5b against a standard curve of known TRAP 5b antigen concentrations.

3.2.6 Element analysis

Element concentrations of Ca and P ions were quantified using ICP-MS (Perkin Elmer Optical Emission Spectrometer, Optima 4300DV). This method was chosen as a possible outcome measure to indicate OC resorption *in vitro* as ICP-MS has a detection level of 0.01mg/l for both Ca and P but more importantly the ability to discriminate differences in the levels of Ca and P through the signal to noise ratio; where the concentration of Ca and P can be determined from the Ca and P levels already present in the medium. Culture media from days 3 and 6 were retained and diluted by a factor of 20 in dH_2O (0.2ml sample to 3.8ml dH_2O) for analysis.

3.2.7 Statistics

Statistical Analysis for all outcome measures was carried out using SPSS v.18 software (IBM, USA). Kolmogorov-Smirnov and Shapiro-Wilk normality tests were conducted and all groups were normally distributed. Differences between treatment groups were assessed using one-way analysis of variance (ANOVA) with a post-hoc, Bonferroni test. Relationships between each outcome measure were investigated using the Pearson's correlation test. A p-value of less than 0.05 was considered statistically significant. A total of five samples were used per time point for all conditions.

3.3 Results

3.3.1 SEM

A small proportion of RAW 264.7 monocytes cultured in the presence of RANKL on HA and β-TCP discs differentiated into large multinucleated cells. Many undifferentiated monocytes were also visible (Figure 3.1). RAW 264.7 monocytes cultured in the presence of RANKL formed eroded areas on the CaP materials. From observation, OC on HA samples formed very superficial erosions whereas OC cultured on β-TCP formed more distinct pit-like erosions (Figure 3.2). OC were evident beside the eroded areas on HA (Figure 3.1a) and appeared to be of similar size and shape to the resorbed areas.

3.3.2 Gravimetric analysis

Inter-group and intra-group variation was great for both materials and seeding densities respectively (figure 3.3). Cells were removed, processed for SEM up to the stage of HMDS evaporation and then the sample was weighed. Potential sources of variation with this outcome measure could be as a result of the degree of compaction of the sintered sample (some samples could be more porous than others) or the variation in evaporation of HMDS from the surface of the sample. In general, there was a greater mass loss (dissolution) on materials sintered at 1100°C compared to 1250°C but this was not affected by the addition of cells to the material. There was no statistical correlation with percentage mass change and cell number (seeding density) for HA or β-TCP; $p = 0.406$ (**Table 3.3**) nor any statistical differences between those with cells compared to no cell control. For this reason mass loss was considered not to be a suitable outcome measure for OC resorption.

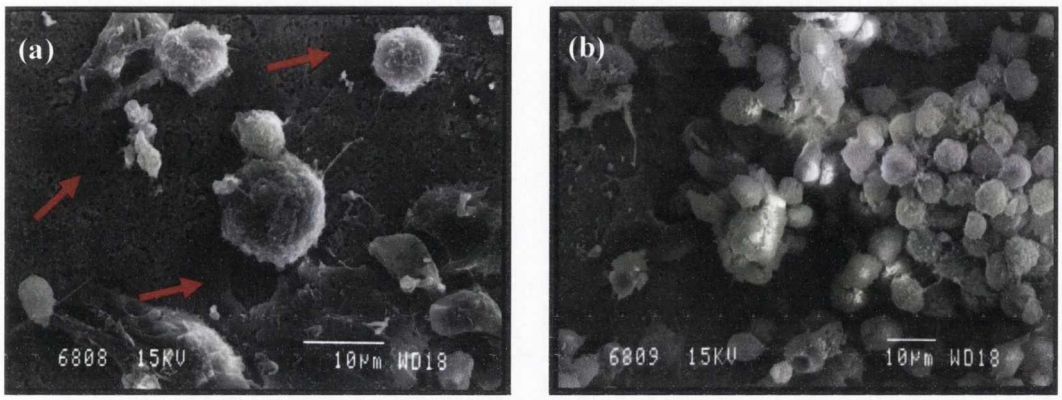

Figure 3.1: SEM micrographs of RAW 264.7 RANKL mediated cells cultured on HA sintered at 1100°C. (a) Seeding density $1x10^5$, magnification x2000, (b) Seeding density $1x10^5$, magnification x1200. Red arrows indicate early stage etched areas beneath the cells. Scale bar is 10µm.

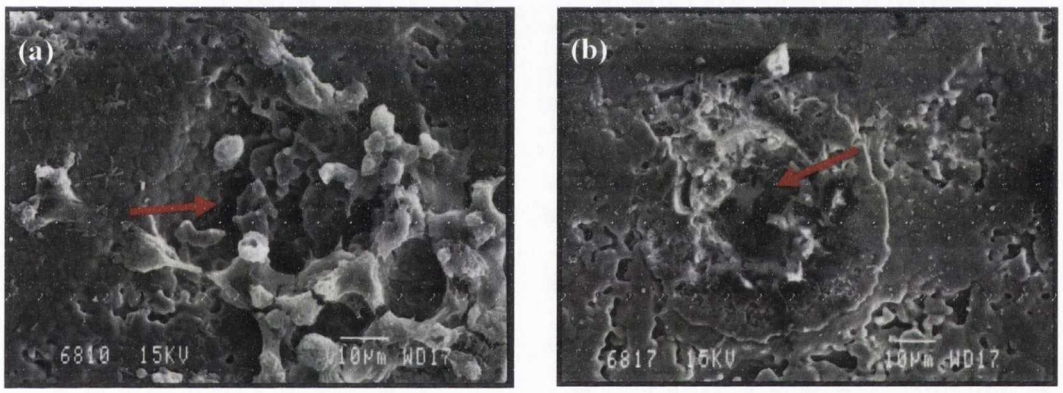

Figure 3.2: SEM micrographs of RAW 264.7 RANKL mediated samples cultured on β-TCP sintered at 1250°C. (a) Seeding density $1x10^5$, magnification x1200, (b) Seeding density $1x10^3$, magnification x1000. Red arrows indicate potential resorption pit. Scale bar is 10µm.

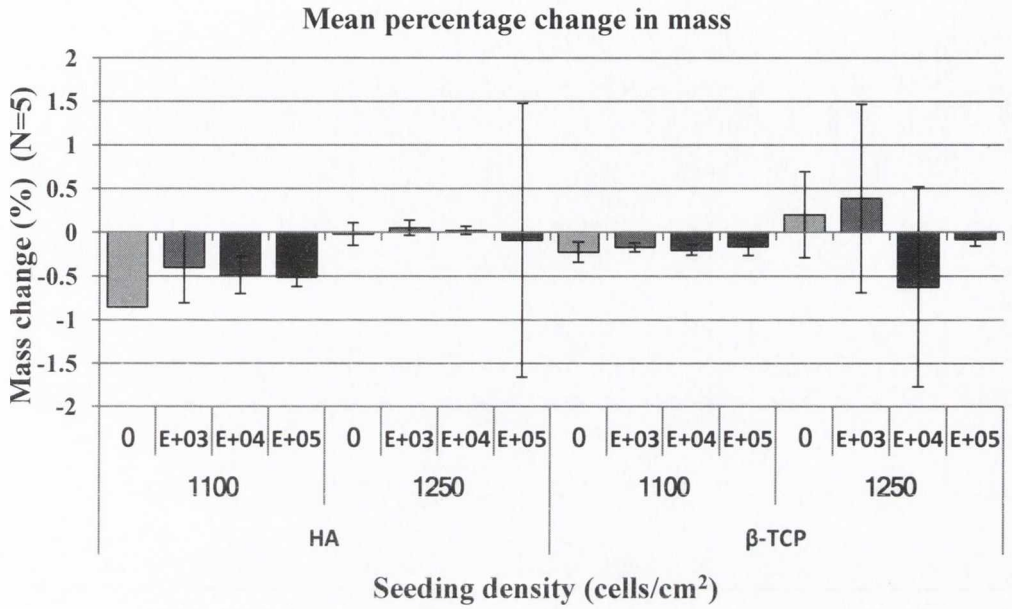

Figure 3.3: Graph showing mean percentage mass change for HA and β-TCP discs after 6 days in cell culture. Error bars represent standard deviation of the mean. Control samples (0) have no cells present. Seeding densities denoted as 1 x E+03, E+04 or E+05 cells/cm^2.

3.3.3 TRAP assay

The release of TRAP 5b enzyme into culture medium was dependent on phase composition and sintering temperature of the ceramic (Figure 3.4). An overall increase in TRAP 5b enzyme release was observed with β-TCP compared to HA and for 1250°C samples compared to 1100°C samples for both HA and β-TCP. A general increasing trend in TRAP 5b enzyme activity is observed with increasing seeding density for both HA and β-TCP, although at the lowest seeding density there was no increase in TRAP5b activity compared to the no cell control. The relationship between TRAP 5b enzyme concentration and cell number was highly significant ($p =<0.001$) with a reasonably strong association ($r = 0.509$) (**Table 3.3**).

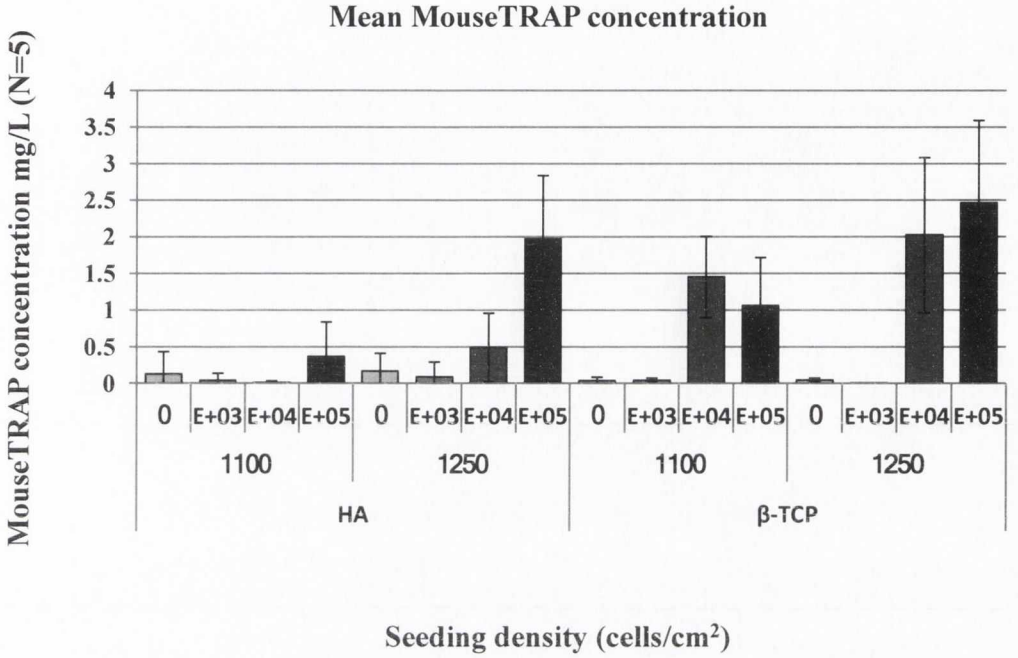

Figure 3.4: Graph showing mean TRAP 5b concentration from RAW 264.7 cells cultured for 6 days on HA and β-TCP. Error bars represent standard deviation of the mean. Control samples (0) have no cells present. Seeding densities denoted as 1 x E+03, E+04 or E+05 cells/cm^2.

3.3.4 Element analysis

The dissolution of Ca and P into culture medium was dependent on phase composition and sintering temperature, similar to the TRAP assay results discussed above (**Figure 3.5**). An increase in Ca and P concentration was observed with β-TCP compared to HA and Ca and P concentration was generally higher for 1250°C samples compared to 1100°C samples for both HA and β-TCP. A general increasing trend in Ca concentration was observed with increasing seeding density for both HA and β-TCP whereas P concentration remained relatively constant for HA and

gradually increased for β-TCP with increasing seeding density. It must be noted that the same trends are observed for control samples in culture medium only.

Strength of correlation between cell number and Ca and P concentration increased from day 3 to day 6 (Ca: r =0.313 and r=0.519 and P: r= 0.121 and r=0.300 respectively). There was also an increase in the significance of these correlations (Ca: p= 0.005 and p<0.001 and P: p=0.283 and p=0.007 respectively) (**Table 3.3**).

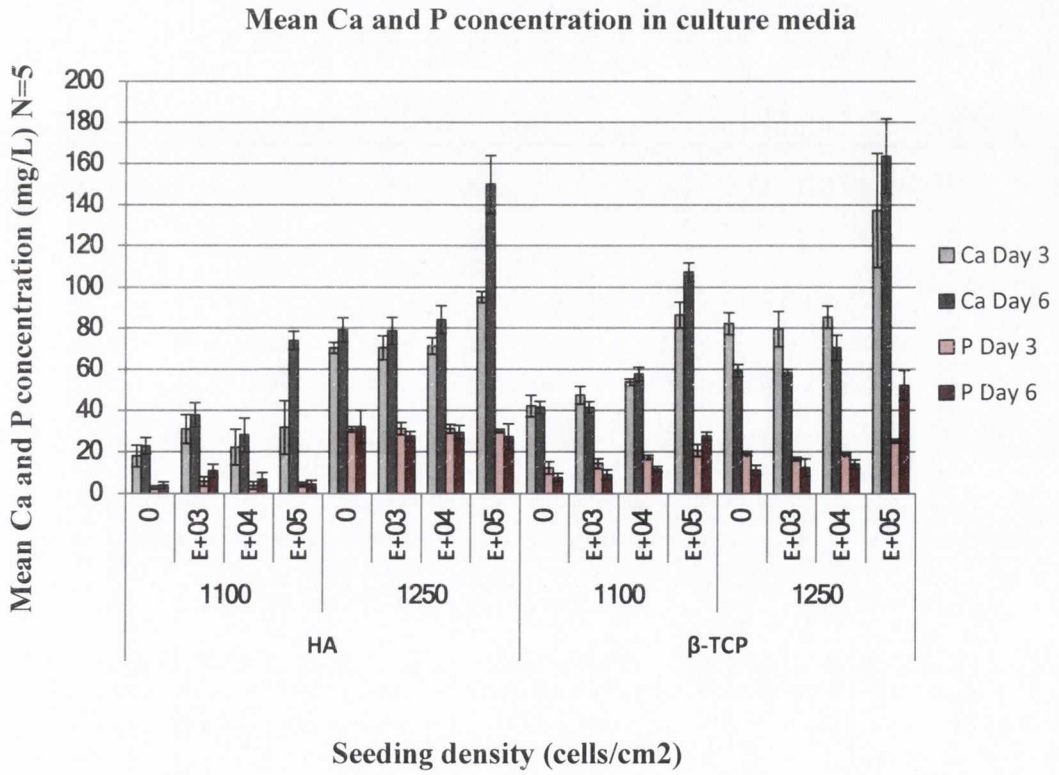

Figure 3.5: Graph showing mean calcium and phosphate concentration for HA and β-TCP cultures after 6 days. Control samples (0) have no cells present. Seeding densities denoted as 1 x E+03, E+04 or E+05 cells/cm^2.

Table 3.3: Correlation table representing statistical analysis carried out on all materials and seeding densities. The top figure in each row represents the Pearson correlation factor (r) showing the strength of correlation and the bottom figure indicates the significance of the results (p-value). For Ca and P results; d3 = day 3 and d6 = day 6.

	Ca d3	P d3	TRAP 5b	Ca d6	P d6	Mass loss
Cell number	0.313	0.121	0.509	0.519	0.3	-0.098
	0.005	0.283	<0.001	<0.001	0.007	0.406
Ca d3		0.726	0.573	0.698	0.783	0.187
		<0.001	<0.001	<0.001	<0.001	0.111
P d3			0.315	0.499	0.761	0.262
			0.004	<0.001	<0.001	0.024
TRAP 5b				0.561	0.493	0.110
				<0.001	<0.001	0.353
Ca d6					0.654	0.04
					<0.001	0.737
P d6						0.145
						0.218

3.4 Discussion

OC are the cells responsible for bone resorption *in vivo* and thus have been selected for use as an *in vitro* model to study the resorption of bone. The selected outcome measures tested were; 1) SEM: to detect OC morphology and pit formation, 2) gravimetric analysis: to see if OC resorption could be related to sample mass loss, 3)

ICP-MS: to quantify mineral release from materials into culture and 4) TRAP 5b enzyme activity: to relate OC activity with resorption. HA and β-TCP materials were used to compare cellular resorption activity with material phase composition. Two sintering temperatures (1100°C and 1250°C) were tested for HA and β-TCP to determine if differences in sintering temperature affect OC formation and activity. Multinucleated cells were derived from the murine monocytic cell line, RAW 264.7, and were seeded at three different densities on the respective materials to assess the effect of OC cell number. A cell line was chosen for the *in vitro* model on the basis of the following;

- Cell lines are more robust and easier to culture than primary cells,
- RAW 264.7 cells require only RANKL stimulation to generate OCs making this model less expensive and more convenient than primary cells which require stimulation with two growth factors,
- Cell lines are more stable and don't have the problems associated with inter-donor variation that are inherent in primary cell assays.

Discs made by hydraulic die compaction were viewed by SEM. Although the discs were 100% HA or 100% β-TCP, the surface morphology under SEM showed that the material surface was not completely smooth and contained some pit-like erosions. This made, it difficult to distinguish these from OC resorption pits and highlighted that ideally a smoother surface would be required to ascertain whether the pits viewed under SEM were actually resorption pits and not pores.

Notwithstanding this, it appeared that SEM micrographs showed early signs of pit formation on both HA and β-TCP ceramics. OC on HA samples formed very superficial pit-like erosions whereas OC cultured on β-TCP formed more distinct pit-like areas (~50μm diameter). OC were evident beside eroded areas on HA and were of similar size and shape to the resorbed areas but few OC remained adherent to the β-TCP substrate after fixation. The surface of HA may have been rougher than that of β-TCP, promoting a favourable surface for OC attachment [97].

As previously discussed, controversial results for the resorption of HA *in vitro* have been reported with some authors observing OC resorption of HA [95][64] and others not [96]. It has been well documented that the solubility of ceramics can influence their resorption *in vitro* [95][64][96] TCP ceramics have been suggested to be unsuitable for OC resorption as they cause a high calcium release into the surrounding media and at the OC-substrate interface due to their high dissolution compared to that of HA [95][96]. The high calcium concentrations are said to inhibit resorptive activity of OC, which subsequently detach from bone [95][96]. It may be reasonable to suggest this phenomenon for the lack of OC cells attached to β-TCP as well as the fact that they were more liable to wash off during fixation.

Continuous resorption trails leading from individual eroded areas by migrating OC that have been reported by others [123][124] were not found on either HA or β-TCP. OC may have resorbed for a period of time before detaching and migrating away from the original site of resorption or in fact the OC may have only begun resorptive activity at the end of the culture period as 6 days is a relatively short time [120].

It was observed that differentiated RAW cell cultures produced cells slightly smaller than those generally associated with mature OC (30μm+ for primary cells in culture[124]). Reasons for this may be: 1) the absence of a purification step (cells

may have been seeded effectively but the purity of the cell population would not have been exclusively OC) and 2) inter-batch variation of RANKL bought from different suppliers may result in differences in batch efficacy (although we referred to a published protocol [56] others have reported using values 1½ times that of the amount used in the present study) [73] [125]. Furthermore, OC formation and activity is enhanced in more acidic environments [126]. The pH of the media in this current experiment was not recorded and this may be a contributing factor to the efficiency of OC formation.

The sealing zone is thought to be the most important ultrastructural feature of the OC, functioning as a determinant for OC phenotype; as OC have very similar morphology to giant cells from the immune system [97]. SEM micrographs (Figure 3.1) did not seem to display the apico-basal morphology associated with cell attachment and sealing zone formation [31][127]. The cells may have been in a 'detachment status' [64] and about to migrate hence they were not spread over the surface of the CaP material or in fact few mature OC may have formed.

In the present study 1250°C samples show increased ion release (calcium and phosphate) and increased TRAP 5b activity compared to their respective 1100°C samples. Results for ICP-MS and the colorimetric TRAP 5b assay showed good correlation with each other ($r = 0.573$ (day3), $r = 0.561$ (day 6)) and with cell number ($p = <0.001$ for both day 3 and day 6). One would predict that samples sintered at a higher temperature would be more thermodynamically stable than those sintered at a lower temperature and in effect have a lower ion release. The opposite of this effect was observed in this study with more mineral being released into the culture media at higher temperatures. To give a greater understanding of the material phase composition of the different samples X-ray diffraction (XRD) analysis could be conducted. This technique is used to determine the crystallinity and phase

composition of a material. For ceramic materials such as HA and β-TCP phase changes can occur after a threshold sintering temperature has been reached. In general, an increase in sintering temperature promotes phase transformation in the order HA > β-TCP > α-TCP. For example, if β-TCP exceeds 1250°C the β-phase can change to alpha (α). α-TCP is more soluble then β-TCP thus if changes in phase composition did occur, this may have increased the solubility of the material and may help explain the results. Also, since higher local Ca^{2+} concentrations inhibit OC resorption then perhaps the more soluble 1100°C materials inhibited OC activity because they dissolve more readily.

Although results indicate high levels of calcium in cell culture media it is difficult to fully interpret the true effect of OC degradation. Calcium concentrations within culture media are hard to quantify as various factors affect the global calcium concentration;

- Ceramics have different calcium ratios,
- Some calcium may have reprecipitated back onto the material,
- Although the seeding density is consistent, the number of mature active OC within the cell population may not be the same [64].

As OC are known to secrete TRAP5b enzyme [127], it was reasoned to use the concentration of TRAP 5b released in culture medium as a possible model for bone resorption by OC *in vitro*, and thus be used as a suitable outcome measure to predict the rate of OC resorption of CaP materials *in vivo*. Together with the ICP-MS results it was concluded that TRAP5b enzyme activity may be a possible outcome measure to predict the rate of osteoclast resorption *in vitro*.

3.5 Summary

The present study has shown that OC generated from RAW 264.7 cells attach to and show potential to degrade HA and β-TCP ceramics *in vitro*. Evidence of TRAP 5b enzyme secretion, mineral release and pit-like formation together provide the basis of this conclusion. To interpret the results fully, quantification of pit number and pit area are required in combination with material property analysis such as phase composition and surface roughness. To conclude, there is potential for the use of RAW 264.7 cells as an *in vitro* model for the osteoclastic resorption of CaP materials.

From this preliminary study, modifications for subsequent protocol development include:

- It will be necessary to quantify resorption pit number and pit area using light microscopy in order to compare these results with the other outcome measures described above.
- The concentrations of RANKL should be optimised to see if OC grow bigger in size and become more active, forming more resorption pits.
- Record the pH of the culture medium as an indication as to whether it is optimum for OC activity.
- Vary the length of the culture period.
- Try to obtain a smoother surface finish for the ceramic discs to make it easier to distinguish OC specific resorption pits.

CHAPTER FOUR

A comparison of osteoclast differentiation and function using osteoclast precursor cells and Raw 264.7 cell line

4 A comparison of osteoclast differentiation and function using osteoclast precursor cells and RAW 264.7 cell line

4.1 Introduction

The aim of the work presented in this chapter was to compare the differentiation and function of OC cells from two sources, murine non-adherent bone marrow osteoclast precursor cells (OPC) and the RAW 264.7 murine monocyte cell line. In an attempt to culture adequate numbers of functionally mature OC, this chapter revisits the culture conditions of Chapter 3 and compares OC differentiation and function of RAW 264.7 cells with Primary OPC. This chapter is comprised of two experiments; each providing subsequent modifications to the protocol as suggested in the summary section of Chapter 3. Experiment A investigates the initial cell culture conditions with a primary focus on RANKL dose and cell culture period.

In a bid to further optimise the reactivity of CaP bone substitutes, a simple thermal treatment was conducted by Egli and co-workers [128] on CaP granules, in which the removal of surface defects had a subsequent reduction in chemical activity of CaP, without inducing significant changes in physical properties. Results indicated that cultures on thermally treated (calcined) β-TCP displayed significantly higher OC specific enzyme activity compared to cultures on non-calcined β-TCP. Thus, the aim of Experiment B was to utilise the cell culture conditions in Experiment A to examine the *in vitro* OC response to dense, polished CaP surfaces before and after calcination.

This work was carried out on placement with the Group for Bone biology, University of Bern, Switzerland and the Robert Mathy's Foundation (RMS), Bettlach, Switzerland under the supervision of two renowned Professors in the fields of Bone

Biology (Prof. Willy Hofstetter) and skeletal materials (Prof. Marc Bohner) respectively.

4.2 Experiment A

Experiment A investigated the effect of RANKL dose on the differentiation of OPC and RAW 264.7 cells grown on tissue culture plates. RANKL was added to cultures at a final concentration of 0, 1, 5 or 20ng/ml. OPC cultures were grown for 6 days and RAW 264.7 cultures for 5 days. The timepoints used for analysis were days 3, 4 and 5 for RAW 264.7 cells and days 4, 5 and 6 for OPC. OC phenotype was characterised by the expression of TRAP and the number of multinuclear cells (3+ nuclei) was counted. N=4 per time point. OC number and activity were assessed by spectrophotometric methods using a cell proliferation and TRAP activity assay respectively. N=6 per timepoint.

4.2.1 Materials and Methods

4.2.1.1 Cell culture of RAW 264.7 cells

RAW 264.7 cells (ATCC) at passage 14 were routinely cultured under standard conditions (37°C, 5% CO_2/95% air) in alpha-MEM (α-MEM), supplemented with 1% (v/v) penicillin/streptomycin (both GIBCO, Invitrogen, UK) 10% (v/v) FBS, (Sigma-Aldrich, UK) and 1% vitamin-C (5mg/ml) (Merck, Middlesex, UK). At day 0, cells were seeded at a density of $2.5x10^4$ cells/cm^2 in tissue culture plates. To initiate differentiation, RANKL (Peprotech, USA) was filter sterilised through a 0.2μm filter and added to complete culture medium at a final concentration of either

0, 1, 5 or 20ng/ml. Culture medium and RANKL was replaced on day 3. Cultures were maintained for 5 days. N=4 for TRAP staining and N= 6 for TRAP activity and XTT.

4.2.1.2 Protocol for murine bone marrow OPC extraction

The mouse was sacrificed by placing in a chamber to which CO_2 was slowly added slowly. The mouse was left inside the chamber for 3 minutes before removing and placed into a beaker of 70% isopropanol to stick down the hair. The mouse was then placed on a cork board covered with cling film. With scissors, the abdomen skin was cut open vertically and horizontally down through the legs and a blade was used to remove muscular flesh to free the bone. One femur and tibia were removed and placed into a beaker with 10ml HANKS* balanced salts solution. The femur was cut at the greater trochanter to leave an open-ended cross-section. The other femur and tibia was then removed and placed into 10ml HANKS. Using fresh instruments (tweezers and blade), the remaining attached muscle and joint capsule were removed. The cleaned bone segment was placed into a fresh beaker containing 10ml HANKS. This process was repeated for the remaining femur and both tibia. A 10 syringe with needle attached was filled with HANKS solution. Whilst holding the specimen with tweezers the needle was inserted into the bone marrow. Initially his was done by placing the needle into the knee joint for both femur and tibia. The medium was then syringed through the bone and collected into a Falcon tube. The cell extraction was then repeated from the other end of the segment (either hip or ankle). The cell extraction procedure was repeated for the remaining 3 bone segments. Once cell extraction was complete the cell suspension was centrifuged for 5 minutes at 1200rpm and the supernatant was poured off the supernatant. The cell pellet was then resuspended in 30ml α-MEM (Resuspended first in 2ml then made up to 30ml). CSF-1 was added to the resuspension at a concentration of 30ng/ml. Cells were

counted with TURKS solution: 2% Glacial Acetic Acid (2ml) in 98ml distilled water with a drop of gentian violet stain (90µl TURKS : 10µl cell suspension, 1:10 dilution). To plate the cells, 15ml of the bone marrow cell suspension was added to a T75 cell culture flask and placed in a 37°C incubator for 24 hours. After 24 hours, the flask was removed from the incubator and non-adherent cells were collected for plating.

* When carrying out BMC extraction HANKS balanced salts solution (low bicarbonate medium) was used to ensure pH stays constant within the medium throughout the length of time required to dissect all bone segments required (normal medium tends to alkalinise with time).

4.2.1.3 Cell culture of primary OPCs

Bone marrow was flushed from the long bones of C57BL/6 mice (7 weeks old) as per Section 4.2.1.2 and cultured for 24h in α-MEM / 1% penicillin/streptomycin (p/s) / 10% FBS in the presence of 30ng/ml colony stimulating factor (CSF)-1 (Chiron, USA). After incubation for 24h, the non-adherent OPC were collected and seeded at a density of $2.5x10^4$ cells/cm^2 in tissue culture plates and cultured for 6 days in the presence of 30ng/ml CSF-1 and either 0, 1, 5 or 20ng/ml RANKL. Culture medium and additives were replaced on day 3. Cultures were maintained for 5 days. N=4 for TRAP staining and N= 6 for TRAP activity and XTT.

4.2.1.4 Cell proliferation

Cell number was determined by measuring viable cells using a cell proliferation kit (Cell Proliferation Kit II [XTT]; Roche Diagnostic). XTT is a tetrazolium salt (yellow) that converts to a formazan dye (orange) in the presence of metabolically active cells. An increase in the number of viable cells directly correlates with an

increase in the amount of formazan formed as measured by absorbance. OC ($2.5x10^4$ cells/cm^2) were cultured in 96-well tissue culture plates. On days 4, 5 and 6 (OPC) and days 3, 4 and 5 (RAW 264.7) cultures (100µl) were incubated for 4 hours at 37°C with 50µl XTT labeling solution (final XTT concentration 0.3mg/ml), and the resulting product was measured spectrophotometrically using a microplate reader (BIO-RAD, USA) at 470 nm. N=6 for all groups.

4.2.1.5 Osteoclast TRAP staining and activity

OC differentiation was determined using a TRAP staining kit (386A, Sigma-Aldrich). Day 4, 5 and 6 (OPC) and day 3, 4 and 5 (RAW 264.7) cultures were fixed in a citrate/acetone solution for 30 seconds, washed in dH_20 and air dried for 15 min. Cultures were then covered in a solution containing napthol AS-BI phosphoric acid and fast garnet GBC salt and incubated for 10 minutes (OPC) and 30 minutes (RAW 264.7) at 37°C in the dark. Cultures were rinsed with dH_2O for 3 min and allowed to air dry before viewing under light microscopy at x10 magnification. The surface area (SA) analysed/well was $110mm^2$. TRAP positive OC were calculated from the mean of 4 wells. An osteoclast was denoted as having three or more nuclei.

OC TRAP enzyme activity was measured by the conversion of p-nitrophenyl phosphate (p-NPP) to p-nitrophenol (p-NP) in the presence of sodium tartrate. On days 4, 5 and 6 (OPC) and days 3, 4 and 5 (RAW 264.7) a separate set of cells were lysed with 100µl lysis buffer (1M NaCl and 0.1% Triton-X 100) and frozen. Once thawed, 50 µL of the cell lysate was transferred to a new well-plate in duplicate. 50µL of 10mM p-NPP in buffer solution (40mM sodium tartrate dehydrate, 50mM Acetic acid 100%, brought to pH 4.8 with sodium hydroxide (NaOH)) was then added to the cell lysate and incubated at room temperature for 45 min. The reaction was stopped with 50µL 0.2M NaOH (All reagents were from Sigma-Aldrich).

Optical absorbance was read at 405nm on a microplate reader (BIO-RAD, California, USA) and TRAP activity was quantified against a standard curve (p-NP). N=6 for all groups.

4.2.2 Experiment A Results

4.2.2.1 TRAP activity

TRAP activity was dose-dependent for both RAW 264.7 and OPC cultures. An increase in RANKL concentration increased TRAP activity, with 20ng/mL RANKL yielding the highest TRAP activity levels for both RAW 264.7 (~1.85 Absorbance Units (AU)) and OPC (~1.6 AU) cultures.

RAW 264.7 cells displayed a time-dependent increase in TRAP activity for all RANKL concentrations (0, 1, 5, 20ng/ml) with an increase in TRAP activity from Day 3-4 and a decrease in activity at Day 5. OPC TRAP activity levels continued to increase with time for all RANKL concentrations with highest TRAP activity levels recorded at Day 6.

At Day 4 (0ng/ml RANKL), RAW 264.7 cells produced ~1 AU compared to ~0.1AU for OPC cultures. This high level of absorbance without RANKL stimulation indicates that TRAP activity alone may not be a suitable outcome measure of RAW OC formation. An OC count is required to achieve more conclusive results with regards OC differentiation.

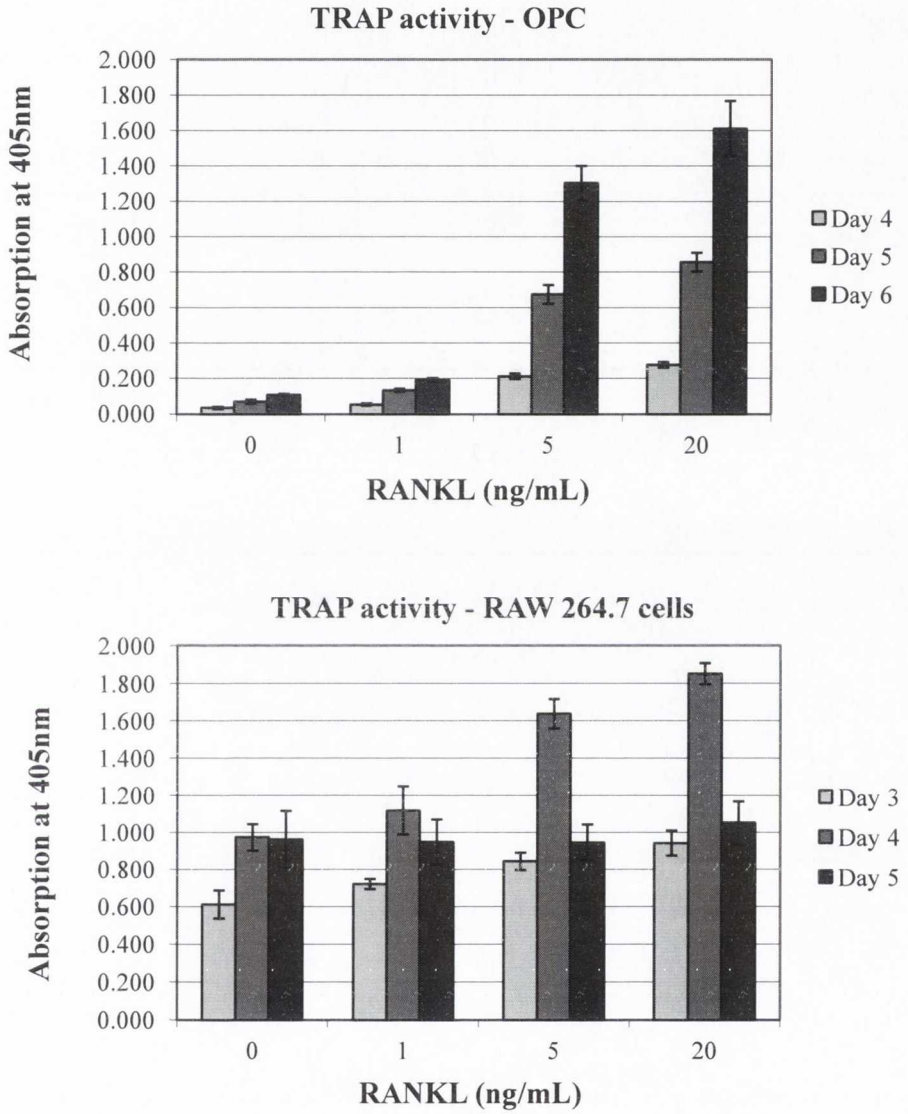

Figure 4.1: TRAP enzyme activity measurements for OPC and RAW 264.7 cells. OPC were cultured for 6 days and RAW 264.7 cells cultured for 5 days.

4.2.2.2 TRAP staining

TRAP positive OC counts were dose-dependent for both RAW 264.7 and OPC cultures. An increase in RANKL concentration increased the number of TRAP positive OC, with 20ng/mL RANKL yielding the highest TRAP positive OC counts for both RAW 264.7 (~267 OC) and OPC (~73) cultures.

No TRAP positive OC were recorded at 0ng/mL RANKL (for both RAW 264.7 and OPC cultures) or 1ng/mL RANKL (OPC cultures only); contrary to the levels of absorption for TRAP activity measurements.

Similar to TRAP activity results, RAW 264.7 cells displayed a time-dependent increase in the number of TRAP positive OC with an increase in the number of TRAP positive OC from Day 3-4 and a decrease at Day 5. OPC TRAP positive OC counts continued to increase with time with the highest number of TRAP positive OC recorded at Day 6.

4.2.2.3 XTT assay

With increasing RANKL concentration there was a general decrease in the number of metabolically active cells (reduced XTT absorption) for both OPC and RAW 264.7 cells at each timepoint. This was expected as RANKL stimulation increases cell differentiation rather than proliferation. There was however a general increase in XTT absorption with time for both RAW 264.7 and OPC cultures for each RANKL concentration. This can be attributed to normal cell culture patterns where cells proliferate and become more confluent with time.

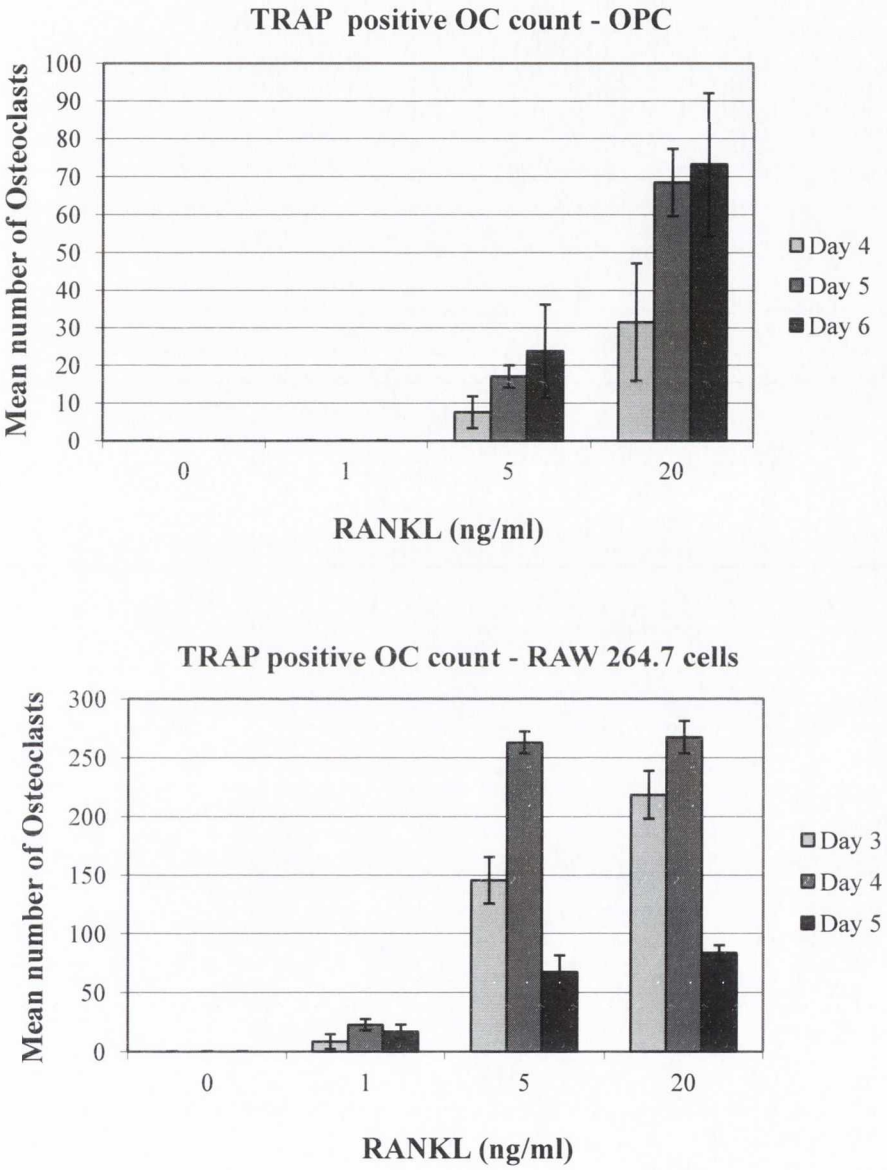

Figure 4.2: TRAP positive osteoclast counts for OPC and RAW 264.7 cells. OPC were cultured for 6 days and RAW 264.7 cells cultured for 5 days. An osteoclast was counted as having 3+ nuclei.

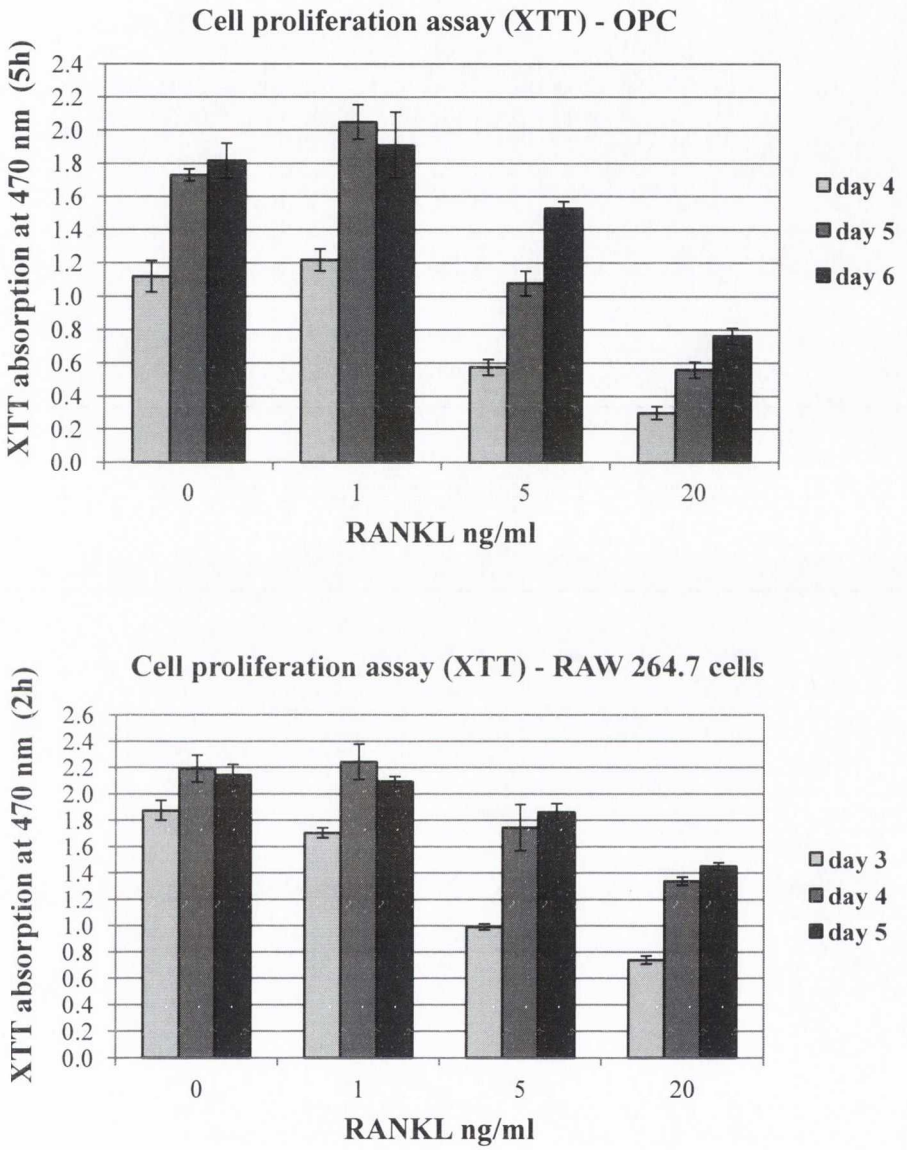

Figure 4.3: Cell proliferation assay for OPC and RAW 264.7 cells. Cell number was determined by measuring viable cells using a cell proliferation kit.

4.2.2.4 Summary of results

- RAW 264.7 cells cultures did not produce TRAP positive OC on Days 3, 4 and 5 at 0ng/mL RANKL. This is in contrast to the levels of absorption seen in TRAP activity measurements.
- OPC also showed small absorbance levels for TRAP activity with 0-1ng/ml RANKL concentrations for Days 4, 5, and 6. However, OC were not formed until addition of 5ng/ml RANKL.
- At the optimum TRAP activity levels for RAW 264.7 cells (Day 4, 20ng/ml RANKL) and OPC (Day 6, 20ng/ml RANKL) there was little difference in TRAP activity at ~0.25AU. However, if we look at cell number for Day 4 at 20ng/mL RANKL concentration it is clear that RAW 264.7 cells produce ~4 times more OC than OPC (267 RAW 264.7 : 69 OPC).
- High background levels of TRAP activity aside, the results and trends for RAW 264.7 and OPC remained consistent:
    - 20ng/mL RANKL produced maximum TRAP activity for both RAW 264.7 cells and OPC
    - There is a reduction in both TRAP activity and OC number at Day 5 for RAW 264.7 cells.
    - There is a continued increase in TRAP activity and OC number for OPC.

4.2.3 Experiment B

Experiment B investigated the differentiation of OPC and RAW 264.7 cells grown on calcined or non-calcined β-TCP. Results from Experiment A suggested a RANKL dose of 20ng/ml RANKL was optimum for both OPC and RAW 264.7 cells. Experiment A also indicated that a longer culture period may be required to allow sufficient time for resorption as OPC had not reached a growth plateau after day 6. With that OPC and RAW 264.7 cultures were grown for 10 days. The timepoints used for analysis were days 2, 4, 6, 8 and 10 for OPC and days 6 and 10 for RAW 264.7 cells. OC phenotype was characterised by the expression of TRAP and DAPI (4',6-diamidino-2-phenylindole) nucleic acid staining. The number of multinuclear cells was counted at days 4, 6, 8 and 10. OC actin ring formation was characterised by the expression of cytoskeletal phalloidin, detected using fluorescence microscopy at days 4, 6, 8 and 10. Resorption at days 6, 8 and 10 was assessed by gold sputter coating with light microscopy. OC activity was measured by TRAP activity on days 4, 6 and 10 (OPC cultures only). Surface properties were determined by SEM. N=6 for test samples and N=3 for no RANKL and no cell controls.

4.2.4 Materials and Methods

4.2.4.1 Preparation of ceramic plates

Dense β-TCP cylinders (Ø30 x 30mm) were supplied by Michel Descamps, Université de Valenciennes, Maubeuge, France. Sample density was close to 98-99% with a grain size close to 1μm. The method of production used by Michel to produce dense β-TCP samples is outlined below.

Synthesis of β-TCP powder

TCP powder was prepared by an aqueous precipitation technique using a diammonium phosphate solution $(NH_4)_2HPO_4$ (Carlo Erba, France) and a calcium nitrate solution $Ca(NO_3)_24H_2O$ (Brenntag, France). The solution pH was adjusted to a constant value of 6.5 by a continuous addition of ammonium hydroxide. Temperature was maintained at 30°C and the solution was matured for 24 h. After maturation, the solution was filtered and the precipitate dried at 80°C. The precipitate was then calcined at 750°C and the powder was subsequently ground to break up any agglomerates formed during calcination. The grinding step was carried out by ball milling in a high density polyethylene milling jar and Y-PSZ grinding media for 3 h [129].

Slip preparation and ceramic manufacturing

TCP samples were prepared by a slip casting method. TCP powder (65 wt.%) was suspended in deionised water (dH_20) to form a slurry. To enhance slip stability, a commercial organic defloculant (Darvan C, R.t.Vanderbilt. Co. Inc.) was introduced at 1.5 wt.% of TCP content. After ball milling for 1 h, the slip was poured into a plaster mould. After drying, samples were sintered at 1100 ◦C for 3 h with a heating rate of 5 °C/min. The density of the sintered samples, determined by Archimedes' method was >99% [129].

As supplied, two β-TCP dense cylinders were then cut into 3mm thick slices using a diamond wire saw (Product No 3032, Well Diamond Wire Saws Inc, Switzerland). Each slice was polished on one side with silicon carbide grit 4000 paper (grain size 5μm) followed by a final polish using a 0.05μm alumina and silicon oxide suspension (Buehler, UK). After polishing the cut slices were mounted again in the

diamond wire saw and cut into 3.8 x 3.8mm plates (Figure 4.4). After washing with 70% isopropyl alcohol (Sigma Aldrich, UK) and sterilised by autoclaving at 121°C for 30 min the plates were either used as such (non-calcined) or calcined at 500°C for 24h.

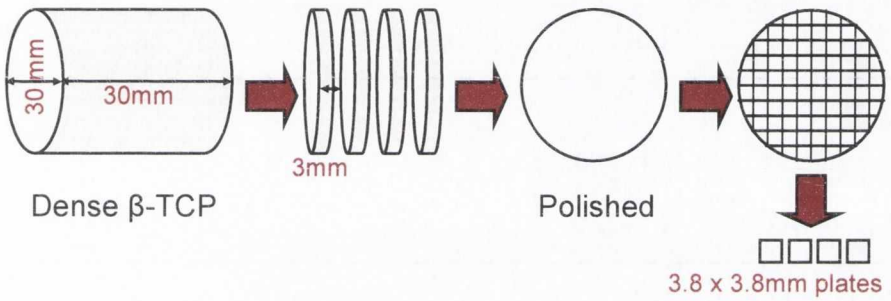

Figure 4.4: Preparation of dense β-TCP plates.

4.2.4.2 Cell culture of RAW 264.7 cells

RAW 264.7 cells (ATCC) at passage 11 were routinely cultured under standard conditions (37°C, 5% CO_2/95% air) in α-MEM, supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen, UK) 10% (v/v) FBS, (Sigma-Aldrich, UK) and 1% vitamin-C (5mg/ml) (Merck, UK). RANKL was filter sterilised through a 0.2μm filter and added to complete culture medium at a final concentration of 20ng/mL. At day 0, cells were seeded onto β-TCP plates at a density of $2.5x10^4$ cells/cm^2 in 96-well plates. Culture medium and RANKL was replaced on days 3, 6 and 8. 15mM hydrochloric acid (HCl) (Sigma-Aldrich) was added to cultures on day 6. Cultures were maintained for 10 days. N=6 for test samples and N=3 for no RANKL and no cell controls.

4.2.4.3 Cell culture of primary OPCs

Bone marrow was flushed from the long bones of C57BL/6 mice (7 weeks old) and cultured for 24h in α-MEM / 1% P/S / 10% FBS in the presence of 30ng/ml CSF-1 (Chiron, USA). After incubation for 24h, the non-adherent OPC were collected and seeded at a density of $2.5x10^4$ cells/cm^2 onto β-TCP plates within 96-well plates and cultured for 10 days in the presence of 30ng/ml CSF-1 and 20ng/ml RANKL. Culture medium was changed on days 3, 6 and 8. 15mM Hydrochloric acid (HCl) was added to cultures at day 6. N=6 for test samples and N=3 for no RANKL and no cell controls.

4.2.4.4 Actin staining of cells

Day 2, 4, 6, 8 and 10 (OPC) and day 6 and 10 (RAW 264.7) cultures were fixed in 3.7% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 10 min, washed in cooled PBS three times and permeabilised over ice in 1% (v/v) Triton X-100 in PBS for 20 min. Cultures were rinsed again three times with cold PBS and stained for 40 minutes at room temperature with AlexaFluor 488 Phalloidin (Invitrogen, UK) dissolved in PBS containing 1% bovine serum albumin (BSA) to label cytoskeletal F-actin. Cultures were then washed with PBS and incubated at 37°C for 5 min with DAPI dilactate (Invitrogen), a nucleic acid counterstain, rinsed three times in PBS and air dried. Cultures were imaged under fluorescence microscopy (Nikon Eclipse E800) at x20 magnification. The SA analysed per field of view was $0.64mm^2$. Based on four fields (SA $2.56mm^2$), 22.5% of a 3.8mm square plate (SA $11.34mm^2$) was analysed. Actin ring number was calculated from the mean of the fields (n=6). Multiple actin rings within an OC were counted individually.

4.2.4.5 Osteoclast TRAP staining and activity

TRAP staining on CaP plates was carried out in accordance with section 4.2.1.5 for day 2, 4, 6, 8 and 10 OPC cultures and day 6 and 10 RAW 264.7 cultures. N=6 for all groups. Cultures were imaged under light microscopy (Nikon Eclipse E800) at x20 magnification. The method for counting the mean number of TRAP positive OC was the same as the actin ring count described in section 4.2.4.4 Sample size was the same at 3.8mm square. TRAP activity, carried out in accordance with section 4.2.1.5, was only carried out on OPC cultures (days 4, 6 and 10) due to sample number restrictions. N=6 for all timepoints.

4.2.4.6 SEM

SEM carried out in accordance with section 3.3.3 was used to view the ceramic surface before and after cell culture with OPC (days 4, 6, 8 and 10) and RAW 264.7 (day 10). N=2 per time point for all conditions.

4.2.4.7 Quantification of resorption

Resorption of the ceramic surface was assessed by gold sputter coating and viewing under light microscopy at x4 magnification for OPC (day 6, 8 and 10) and RAW 264.7 cultures (day 6 and 10). N=4 per time point for all conditions. To prepare samples for sputter coating, they were transferred into a 24-well plate containing 1ml 70% isopropanol (Sigma Aldrich) per well and the plate was sonicated for 5 minutes. Samples were then cleaned one at a time in a petri-dish containing 70% isopropanol. A small brush was used to remove the cells. Samples were air dried before sticking onto stubs for sputter coating. To quantify the percentage area resorbed (Equation 4.1) the point to point method was used. At x4 magnification the total disc surface could be viewed using four rotations of the grided ocular lens. Each grid is divided

into 100 squares. Each point on the grid to come into contact with a resorbed area was counted.

$$\% \text{ area resorbed} = \left(\frac{\text{Number of contact points}}{400}\right) \times 100 \qquad \textbf{Equation 4.1}$$

4.2.5 Experiment B Results

4.2.5.1 Actin staining

Actin staining showed the formation and number of actin rings within OC. Multiple actin rings were found within a single OC for OPC and RAW 264.7 cultures on both calcined and non-calcined β-TCP (Figure 4.5). The majority of actin rings were intact at Day 6 and had fragmented by Day 10 for both OPC and RAW 264.7.

4.2.5.2 TRAP staining and activity

TRAP staining provided a means of visualising mature OC's attached to the β-TCP discs. OC stained positive for TRAP at Days 6 and 10 for OPC and RAW 264.7 cultures on both calcined and non-calcined β-TCP (Figure 4.6). By Day 10 RAW 264.7 OC were much bigger and had more nuclei per OC compared to OC differentiated from OPC. TRAP activity (conducted for OPC cultures only, Figure 4.7) results exhibited a 5 fold increase from Day 6 (~0.5 AU) to Day 10 (~2.5 AU).

4.2.5.3 Quantification of actin ring and osteoclast number

For OPC cultures, OC number increased throughout and exhibited a similar ~5 fold Day 8 (59 non-calcined, 84 calcined) and decreased thereafter (~16 at Day 10) (Figure 4.8). No increase from Day 6 to Day 10(~19 to 100 respectively) as observed with TRAP activity. Actin ring number increased from Day 4 (26 non-calcined, 32 calcined) to significant difference was observed between calcined and non-calcined groups. For RAW 264.7 cultures, OC number increased from Day 6 (9 non-calcined, 12 calcined) to Day 10 (26 non-calcined, 41 calcined) with little difference in actin ring number Figure 4.9. No significant difference was observed between calcined and non-calcined groups.

4.2.5.4 Quantification of resorption

Sputter coated samples were viewed under light microscopy to achieve a colour contrasting surface profile. Dark coloured areas represented unresorbed surface and light coloured areas represented surface resorption (Figure 4.10). RAW 264.7 OC resorbed in a circular pattern similar to the size and shape of RAW 264.7 OC. OPC OC resorbed in a more linear fashion with trail like excavations.

Timepoints tested were Days 6, 8 and 10 for OPC and Days 6 and 10 only for RAW 264.7 cells due to sample number restrictions. No active resorption was observed at Day 6 for either cell group. Resorption significantly increased at Day 8 for OPC and Day 10 for RAW 264.7. At Day 10, ~60% (RAW 264.7) and ~90% (OPC) of the β-TCP surface was resorbed (Figure 4.11). No significant differences were observed between calcined and non-calcined materials for RAW 264.7 or OPC. Highly magnified SEM micrographs (Figure 4.12) illustrate the CaP surface resorption as a function of time. Areas of resorption appear deeper with time.

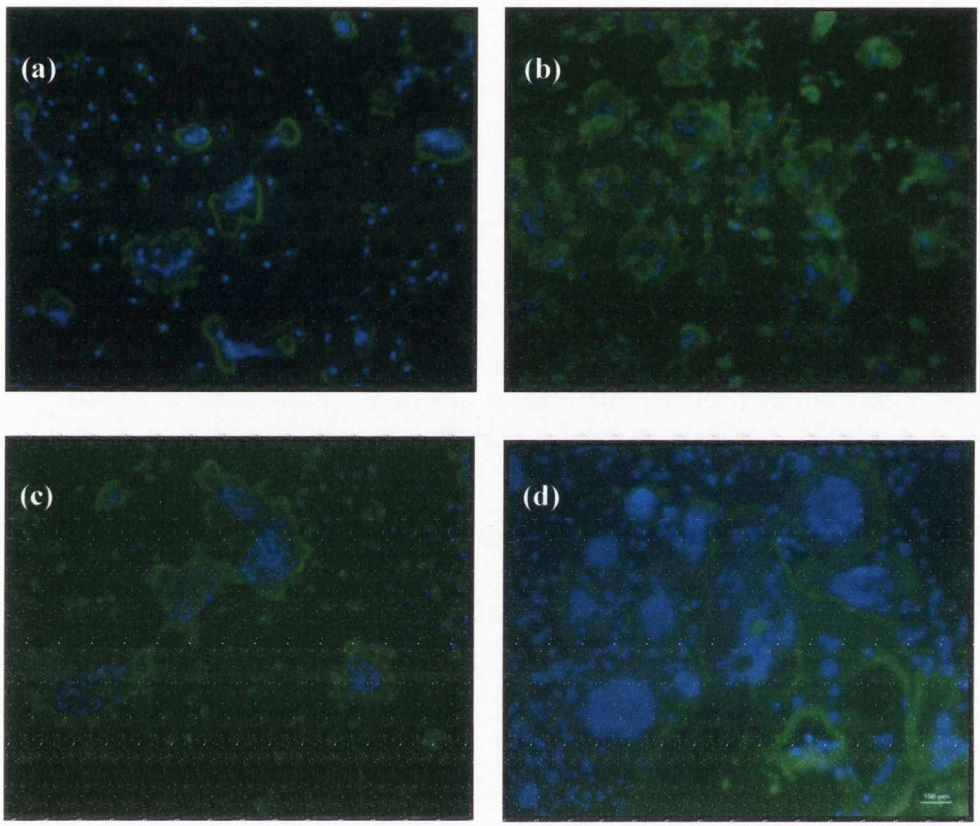

Figure 4.5: Actin Staining of OPC and RAW 264.7 cells on calcined β-TCP. (a) OPC Day 6, (b) OPC Day 10, (c) RAW 264.7 Day 6, (d) RAW 264.7 Day 10. Magnification x20.

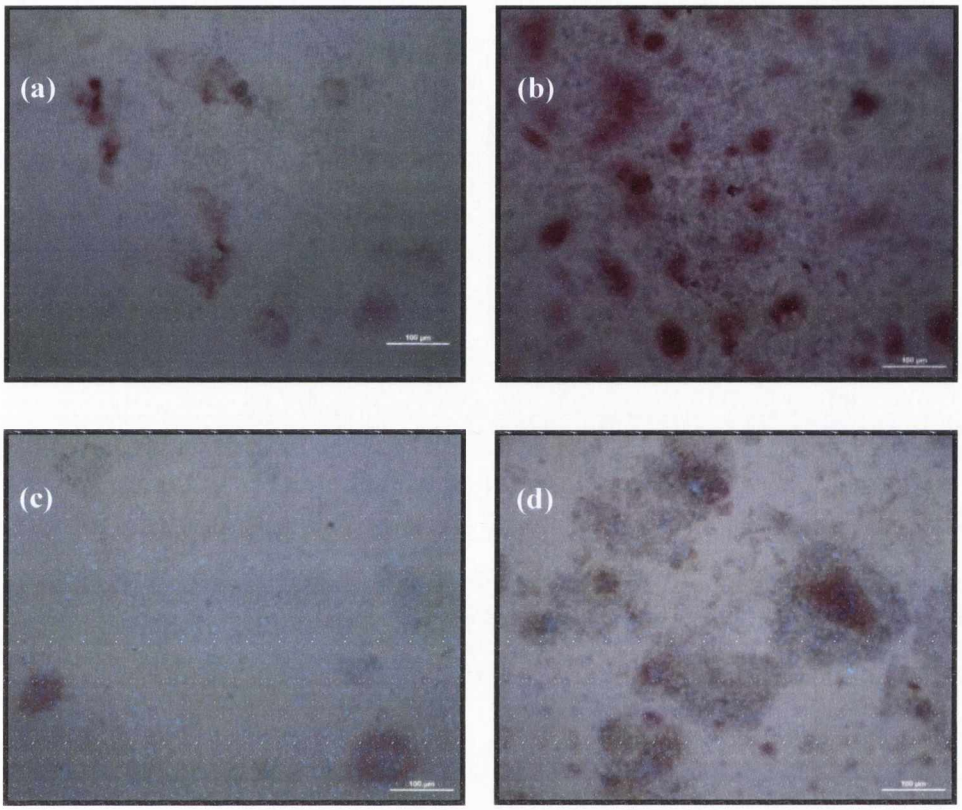

Figure 4.6: TRAP Staining of OPC and RAW 264.7 cells on calcined β-TCP. (a) OPC Day 6, (b) OPC Day 10, (c) RAW 264.7 Day 6, (d) RAW 264.7 Day 10. Magnification x20. Scale bar 100 μm.

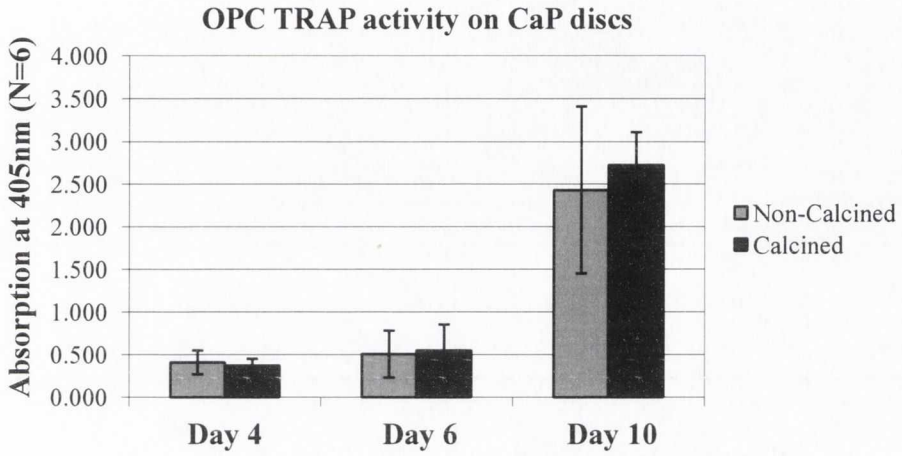

Figure 4.7: TRAP activity of OPC on β-TCP after 10 days.

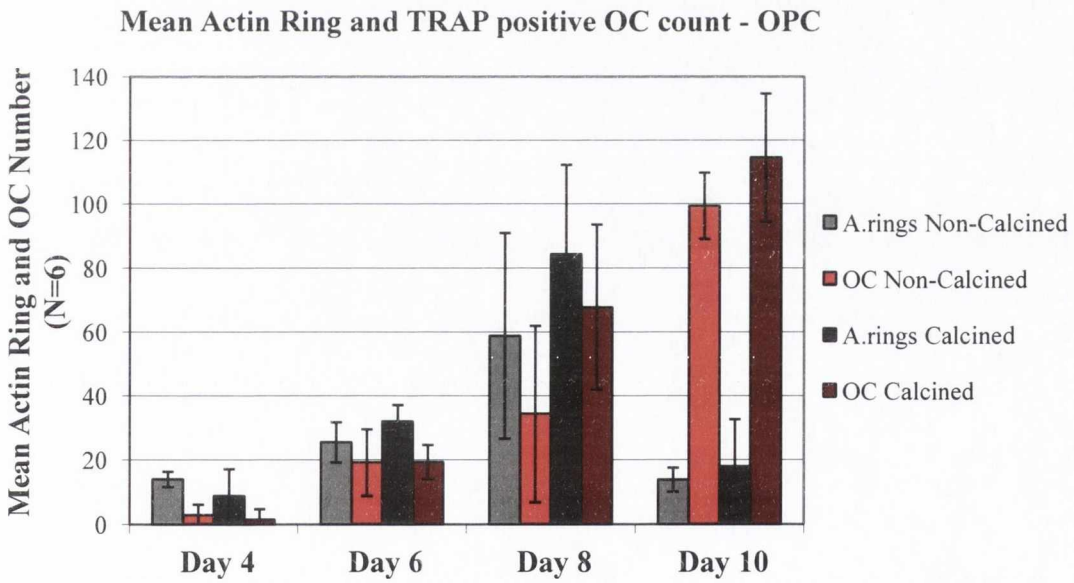

Figure 4.8: Mean Actin Ring and TRAP Positive OC count for OPC.

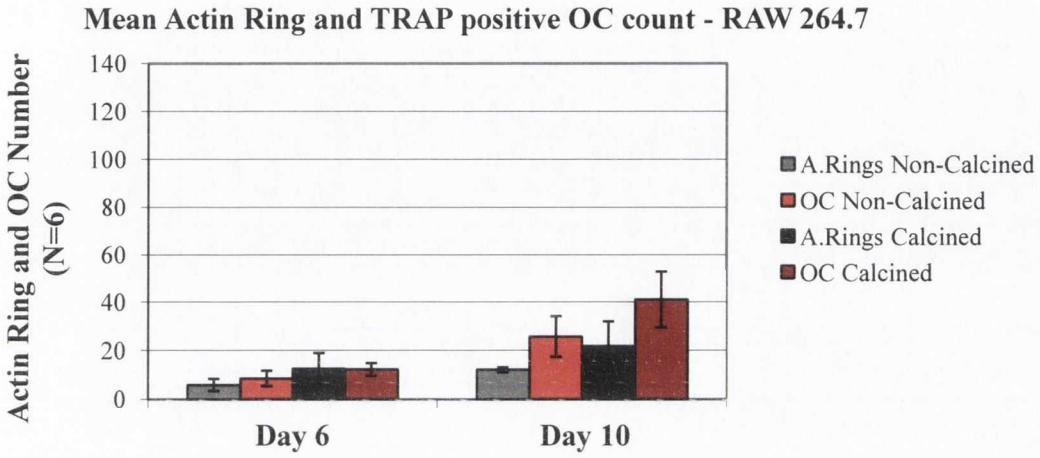

Figure 4.9: Mean Actin Ring and TRAP Positive OC count for RAW 264.7 cells.

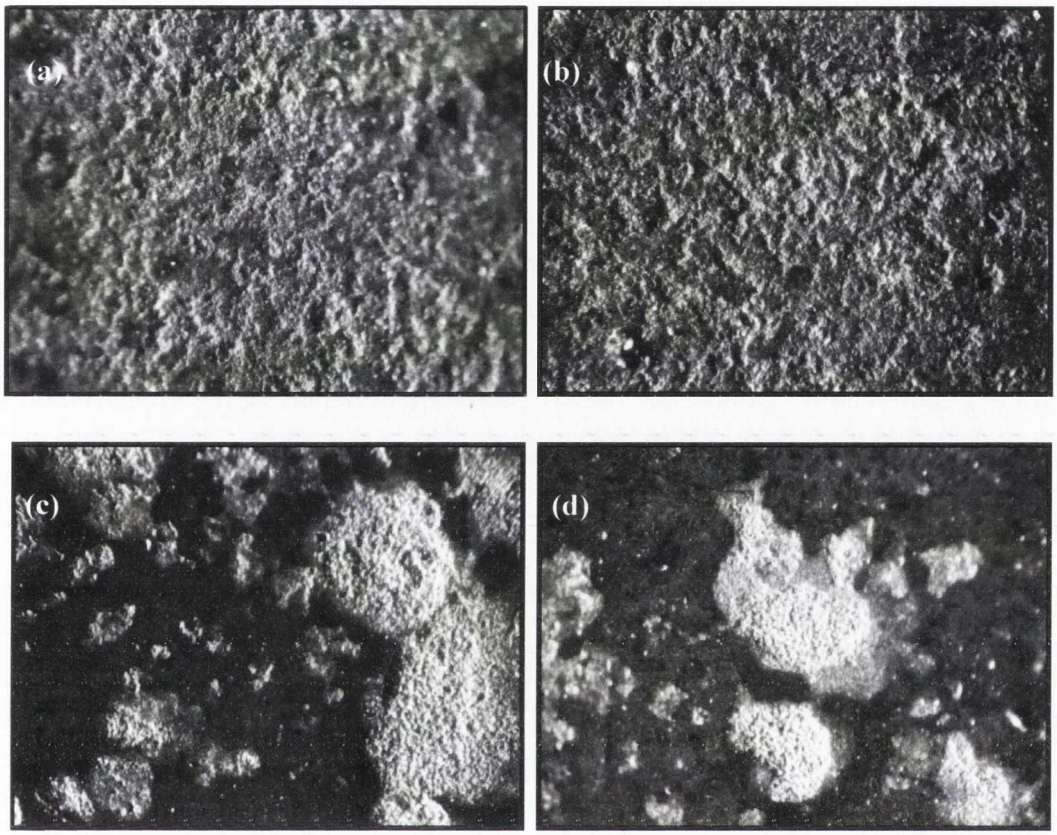

Figure 4.10: Gold sputtered samples of non-calcined and calcined β-TCP after 10 days in culture. (a) OPC non-calcined, (b) OPC calcined, (c) RAW 264.7 non-calcined, (d) RAW 264.7 calcined. Magnification x4.

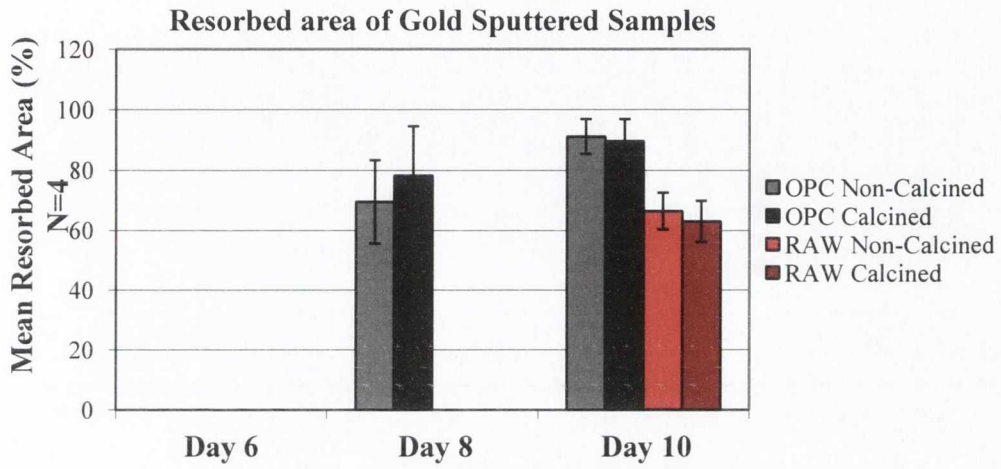

Figure 4.11: Mean percentage area resorbed for calcined and non-calcined β-TCP. Sputter coated samples were viewed under light microscopy and quantified using the point to point method.

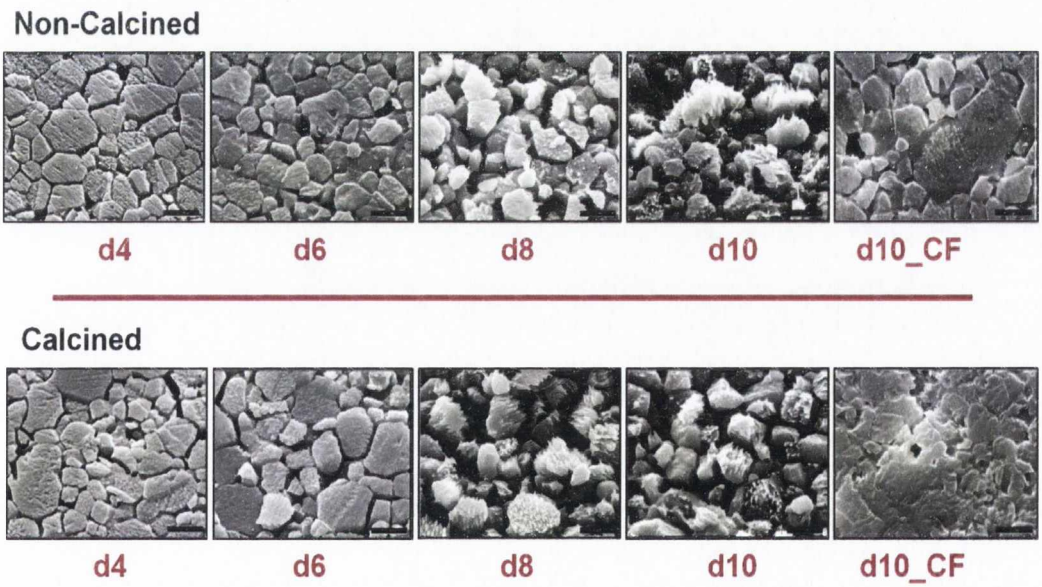

Figure 4.12: SEM images of β-TCP disc surfaces after OPC culture. CF = Cell free controls. (x10,000 magnification, scale bar 2μm).

Table 4.1: A summary of experimental modifications to the initial OC generation assay.

Experimental Modifications	Chapter 3: Development of an OC assay	Chapter 4: A comparison of osteoclast differentiation and function
Cell Type	RAW 264.7	RAW 264.7 and OPC
Media Type	D-MEM	α-MEM
Media Supplements	10% v/v FBS	10% v/v FBS
	4mM L-glutamine	1% Vitamin C (5mg/mL)
	1.0mM sodium Pyruvate	1% v/v PenStrep
	1% v/v antimycotic/antibiotic	
Seeding Density	1x 10^{3-5} cells/cm^2	2.5 x 10^4 cells/cm^2
Additives	RANKL (35ng/ml)	RANKL (0-20ng/ml)
		HCl (15mM) *
		CSF-1 (30ng/mL)**
Culture Period	6 Days	5 Days Experiment A
		10 Days Experiment B
Disc Maunfacture	Powder compaction	Slip cast (dense)
	Sintered (1100°C or 1250°C)	Sintered 1100°C
		(with/without calcination at 500°C)

* Experiment B only **OPC cultures only

4.3 Overall discussion

The aim of this experimental work was to develop functionally active OC *in vitro*. Changes were made to the culture conditions in a bid to optimise the environment for OC differentiation and activity (Table 4.1). This work also examined the *in vitro* OC response to dense, polished CaP surfaces before and after calcination. Calcination of CaP occurs at a lower temperature than sintering in a bid to maintain particle size but to remove surface defects on the CaP, affecting chemical reactivity of the CaP. Sintering by comparison heats the CaP at high temperatures and affects CaP physical properties by binding adjacent particles together, increasing the density of the CaP.

Initial changes to the cell culture conditions in Experiment A showed that TRAP activity and TRAP positive OC counts were dose dependent for both RAW 264.7 and OPC cultures. An increase in RANKL concentration increased TRAP activity and TRAP positive OC counts, with 20ng/mL RANKL yielding the highest TRAP activity levels and TRAP positive OC counts for both cultures. At the optimum TRAP activity levels for RAW 264.7 (Day 4, 20ng/ml RANKL) and OPC (Day 6, 20ng/ml RANKL) there was little difference in activity at ~0.25AU. However, there was a significant difference in cell number with RAW 264.7 cells forming nearly 4 times more OC than OPC (267 OC for RAW 264.7 Day 4 and 73 OC for OPC Day 6). This indicates that RAW 264.7 cells differentiated more than OPC in tissue culture plates.

Experiment A concluded that a RANKL dose of 20ng/ml RANKL was optimum for both OPC and RAW 264.7 cells. Experiment A also indicated that a longer culture period may be required to allow sufficient time for resorption as OPC had not reached a growth plateau after day 6. With that OPC and RAW 264.7 cultures were grown for 10 days in Experiment B. Experiment B also introduced the addition of

15mM HCl to cell cultures. Extracellular acidification has been shown to stimulate bone resorption by extending OC survival and facilitating OC adhesion and migration [130]. As this work was carried out in another lab, their experience in bone cell culture techniques was acknowledged and this was the first time HCl addition to culture medium was considered for the OC generation assay.

Differentiation of cells was induced by addition of 20ng/mL RANKL to culture medium. Both cell sources formed TRAP positive OC cells (Figure 4.6) on calcined and non-calcined β-TCP containing morphological actin rings (Figure 4.5) characteristic of active OC. Both cell sources also formed resorption trails or pits on calcined and non-calcined β-TCP (Figure 4.10). Resorption patterns differ in shape and size and are generally characterised as pits or trails. Resorption pits by RAW 264.7 cells corresponded to the "reticulate patch resorption" and resorption trails by OPC corresponded to "longitudinally extended resorption" as described in [123]. The majority of actin rings were intact at Day 6 and had fragmented by Day 10 for both OPC and RAW 264.7. By Day 10 RAW 264.7 OC were larger and had more nuclei per OC compared to OC differentiated from OPC. TRAP activity was only conducted for OPC and no difference was observed for calcined or non-calcined β-TCP (Figure 4.7).

The same general trends were observed for OPC and RAW 264.7 differentiation and function;

- An increase in TRAP positive OC up to Day 10. There was a ~5 fold increase for OPC and a ~3 fold increase for RAW 264.7 from Day 6-10.
- No resorption pits were formed at Day 6 followed by formation and increase in resorption pit formation up to Day 10.

- No difference was observed between calcined and non-calcined β-TCP for any outcome measure.

, apart from the following:

- Actin ring formation: OPC Mean AR count increased from Day 4-8 and decreased at Day 10 whereas RAW 264.7 Mean AR count had a marginal increase from Day 6 to Day 10.

- Resorption pit shape: OPC resorbed in a linear fashion revealing resorption trails whereas RAW 264.7 resorption appeared more static forming distinct circular resorption pits.

RAW 264.7 cells formed fewer TRAP positive OC and resorbed less percentage surface area compared to OPC. A possible reasoning for this difference is that RAW 264.7 OC are larger than OPC and thus fewer RAW 264.7 OC could theoretically fit into the field of view during quantification by light microscopy. It must be noted that the reduction in number of RAW 264.7 TRAP positive OC relative to OPC is in contrast to Experiment A where RAW 264.7 formed 4 times more TRAP positive OC than OPC by Day 4 on tissue culture plastic. It is known that OC prefer rough surfaces to smooth surfaces [131][31][76] therefore the presence of a rougher β-TCP substrate should not reduce OC formation compared to smoother tissue culture plastic. Perhaps a combination of the interaction of cell culture medium with the surface of the β-TCP and the difference in surface energy of tissue culture plastic compared to β-TCP may have influenced RAW 264.7 OC formation and adhesion. Surface energy and surface chemistry have been shown to affect cell adhesion and cell spreading respectively [132].

Calcination of dense β-TCP had no effect on resorption by the OPC or RAW 264.7 OC *in vitro* or on OC formation and activity. This is in contrast to recent published data [128] where after 5 days there was a significant reduction in OPC TRAP activity for non-calcined β-TCP compared to calcined β-TCP. The study in [128] proposed that a change in surface dissolution capacity of the calcined and non-calcined β-TCP may have altered the OC adhesion and/or migration mechanism. No quantification of OC resorption on calcined and non-calcined β-TCP was reported in the study [128].

The current modifications to the OC assay from Chapter 3 experiments were as follows:

- An increase in the culture period from 6 days to 10 days, testing at 2 day intervals (for OPC only).
- A range of RANKL concentrations (0, 1, 5, 20ng/mL) were tested as opposed to simply using 35ng/mL.
- Addition of 15mM HCl to cell culture medium after Day 6.
- Using α-MEM as the culture medium instead of DMEM.
- Changing the cell seeding density to 2.5×10^4 instead of 1×10^3, 1×10^4, 1×10^5.
- Dense β-TCP compared to compacted β-TCP discs.

Increasing the culture period allowed cells more time to mature and become active and increasing the frequency of testing days allows important events not to be missed. More timepoints were needed for RAW 264.7 cells in order to give a comprehensive overview of OC differentiation and function. Addition of 15mM HCl and the use of α-MEM may have played a role in acidifying the OC extracellular environment in favour of OC formation and activity and using a dense lightly polished substrate made it easier to see resorption pits on flatter surfaces.

4.4 Summary

The present study has shown that OC generated from OPC and RAW 264.7 cells display the same general trends when assessing OC differentiation and function with the exception of resorption pit shape and actin ring number. Calcination of dense β-TCP had no effect on resorption by OPC or RAW 264.7 OC *in vitro* or on OC formation and activity. To interpret the results fully, more timepoints are required to investigate RAW 264.7 cells more comprehensively as an *in vitro* model for the OC resorption of CaP materials.

From this study, modifications for subsequent protocol development include:

- Justification for using 20ng/mL RANKL.
- Justification for the addition of 15mM HCl.
- Justification for the timepoint at which HCl is added to culture medium.
- Recording of pH of the culture medium as an indication as to whether it is optimum for OC formation and activity.

- Investigation of elemental ion release into culture medium and assess the effects on OC activity.
- Investigation of an alternative method for the quantification of resorption pit area.

CHAPTER FIVE

Modifications to the development of an osteoclast assay for the assessment of RAW 264.7 osteoclast cells *in vitro*

5 Modifications to the development of an osteoclast assay for the assessment of RAW 264.7 osteoclast cells *in vitro*

5.1 Introduction

Analyses of Chapter 4 experiments (conducted in Switzerland) highlighted that;

- A different RANKL dose and supplier was used for the generation of OC.
- There was no comparative control for the addition of HCl at Day 6 to assess the effect of acid addition.
- There was no justification for the concentration of HCl added (15mM).
- The number and activity of OC formed from RAW 264.7 cells peaked at day 4. Time points were limited for RAW cells and further investigation is required.
- The gold sputtering and oblique light microscopy technique used for assessing area resorbed is subjective.
- From the SEM images, resorption on samples cultured with OPC displayed uniform attack rather than pitting.

The purpose of this chapter is to address and/or justify the first three bullet points above. Three preliminary studies were designed to further develop the OC assay;

Experiment C: To determine the effect of RANKL dose and supplier on the generation and activity of mature OC.

Experiment D: To determine the effects of HCl addition to culture medium on β-TCP surfaces in a cell-free environment.

Experiment E: To determine the effect HCl addition to culture medium has on culture medium pH during incubation.

5.2 Experiment C

Chapter 4 experiments conducted in Switzerland used 20ng/mL RANKL (PeproTech) for the differentiation of OC. A decision was made to test both RANKL concentration and supplier as the initial protocol in Chapter 3 used 35ng/mL RANKL from R&D Systems. A preliminary experiment carried out in our lab testing the efficacy of R&D systems RANKL for the differentiation of OC found 70ng/mL RANKL to be more effective than 35ng/mL. Therefore the aim of this experiment is to compare the differentiation of OC after addition of RANKL at either a concentration of 20ng/mL (PeproTech), 50ng/mL (PeproTech) or 70ng/mL (R&D Systems).

5.2.1 Materials and Methods

Cell culture of RAW 264.7 cells

RAW 264.7 cells (ATCC) at passage 6 were routinely cultured under standard conditions (37°C, 5% CO_2/95% air) in α-MEM, supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen) and 10% (v/v) FBS, (Sigma-Aldrich). RANKL was filter sterilised through a 0.2μm filter and added to complete culture medium at a final concentration of either 20ng/mL (PeproTech), 50ng/mL (PeproTech) or 70ng/mL (R&D Systems). At day 0, cells were seeded onto 96-well tissue culture plates at a density of $2.5x10^4$ cells/cm^2. Culture medium and RANKL was replaced

on days 3 and 6. Cultures were tested on days 4, 5, 6 and 8. N=6 for all groups.

TRAP Activity

OC TRAP enzyme activity was measured by the conversion of p-nitrophenyl phosphate (p-NPP) to p-nitrophenol (p-NP) in the presence of sodium tartrate. On days 4, 5, 6 and 8 RAW 264.7 cells were lysed with 100µl lysis buffer (1M NaCl and 0.1% Triton-X 100) and frozen. Once thawed, samples were assayed as per section 4.2.1.5 and quantified against a standard curve of p-NP. N=6 for all groups.

5.2.2 Results

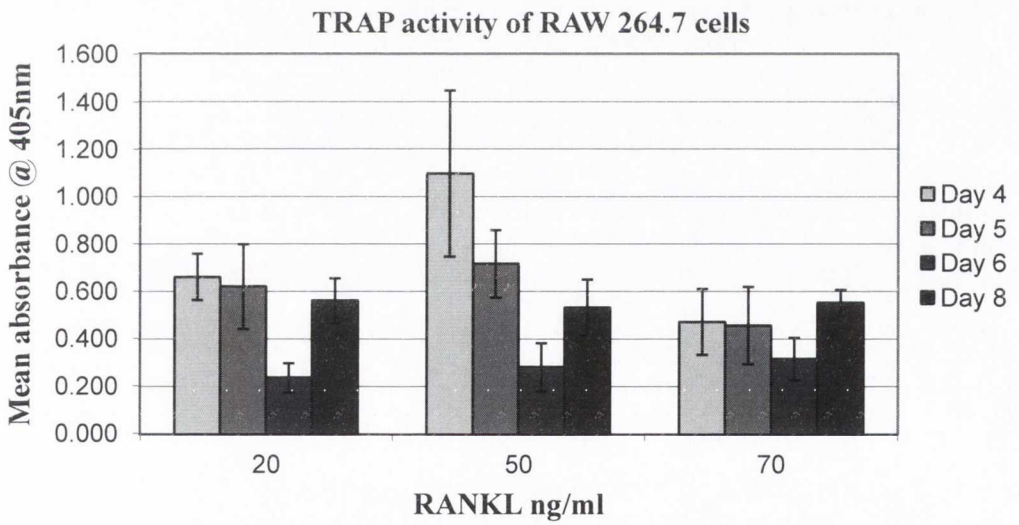

Figure 5.1: TRAP activity of RAW 264.7 cells cultured with various RANKL concentrations

In general, TRAP activity is highest at Day 4 and decreases to Day 6 followed by and increase to Day 8 for all RANKL doses (Figure 5.1). Day 4 50ng/mL RANKL (PeproTech) yields the highest overall activity. There is little difference in TRAP activity between groups at Days 6 and 8. At Days 4 and 5, PeproTech RANKL concentrations (20 and 50ng/mL) yield higher TRAP activity results than the R&D Systems RANKL concentration of 70ng/mL. Taking the error bars into consideration, 50ng/mL RANKL (PeproTech) only produced marginally higher TRAP activity results compared to 20ng/ml RANKL (PeproTech).

PeproTech RANKL indicated that there was more OC differentiation compared to R&D Systems RANKL due to the increased TRAP activity results at lower concentrations. With that and taking the error bars into consideration a decision was made to choose 20ng/mL RANKL as the dose for future experimental work, adopting this as a permanent modification to the initial protocol.

5.3 Experiment D

Chapter 4 experiments conducted in Switzerland also added 15mM HCl to cultures to promote a more acidic environment ideal for the resorptive activity of OC [41]. In order to justify the addition of HCl to the initial cell culture protocol as per Chapter 3 the following cell free experiment was set up to investigate the HCl-material interaction.

The aim of this experiment was to compare the surface of β-TCP discs before and after addition of HCl in a cell free environment. This was to ensure that any pits observed previously on β-TCP discs are a result of cell mediated action rather than from dissolution as a direct effect of the presence of HCl.

5.3.1 Materials and methods

Preparation of β-TCP discs

Dense β-TCP cylinders were produced as described in Section 4.2.4.1. The cylinders were then cut into 4mm thick discs using a diamond wire saw (Struers Accutom-50). Each slice was flattened by manually grinding with silicon carbide paper grit P500 (grain size 30μm) (Struers). Discs were then washed with 70% isopropyl alcohol (Sigma Aldrich) and sterilised by autoclaving at 121°C for 30 minutes.

Discs were placed into 96-well plates and covered in 200μl of α-MEM, supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen) and 10% (v/v) FBS, (Sigma-Aldrich). At Day 0 culture medium contained either, 0mM HCl, 10mM HCl or 15mM HCl and was incubated under standard conditions (37°C, 5% CO_2/95% air). A separate group of samples containing 0mM HCl was incubated at 37°C, 10% CO_2/90% air. All samples (N=5) were incubated for 12 days. Medium with/without HCl was replaced on Days 3, 6 and 9.

Element analysis

Concentrations of Ca and P ions were quantified using ICP-MS (Perkin Elmer Optical Emission Spectrometer, Optima 4300DV). Culture media from days 3, 6, 9 and 12 were retained and diluted by a factor of 80 in dH_2O (0.05ml sample to 3.95ml dH_2O) for analysis (N=5).

Scanning Electron Microscopy

SEM was used to view the ceramic surface before and after incubation with media. Day 12 samples (N=3) were processed for SEM as described in Section 3.2.3.

5.3.2 Results

Scanning Electron Microscopy

SEM analysis of the β-TCP discs showed that no pits were present after incubation with or without HCl (Figure 5.2). Some samples displayed a residue on top of the disc surface. This residue is most likely to be: 1) protein plaques on the disc surface, presumably from crystallisation of proteins in the culture medium or 2) Silicon Carbide remnants from processing the discs, as the β-TCP discs before incubation with culture medium appear similar to the 0mM HCl control samples incubated in culture medium.

Element Concentration

There was a general decreasing trend in Ca ion concentration with time (Figure 5.3). 0mM HCl 10% CO_2 has the highest Ca ion concentration throughout whereas 0mM HCl 5% CO_2 has the lowest Ca ion concentration throughout. This can be attributed to the more acidic nature that results from increasing the percentage CO_2 within the incubator [126]. 10mM HCl 5% CO_2 yields a marginally higher Ca ion concentration compared to 15mM HCl 5% CO_2 however, is not significant due to considerable overlapping of error bars. Per condition there was higher Ca ion concentrations at the earlier time points of Day 3 and Day 6 compared to Days 9 and 12. The reduction in ion concentration with time would indicate reprecipitation onto the β-TCP disc. The order of Ca ion concentration from highest to lowest is: 0mM HCl 10% CO_2, 10mM HCl 5% CO_2, 15mM HCl 5% CO_2 and 0mM HCl 5% CO_2. The same general trends in Ca ion concentration were observed for P ion concentration (Figure 5.4). The level of P ions were approximately half that of the Ca ion concentration.

Assessing the release profiles for 15mM HCl 5% CO_2 (as used in Switzerland Experiments) and 0mM HCl 5% CO_2 (as used in the initial protocol) there is very little difference in Ca ion concentration after Day 3. Combined with the SEM micrographs (Figure 5.2) of the β-TCP surfaces it can be concluded that the pits obtained in Switzerland using 15mM HCl at 5% CO_2 were cell-mediated rather than by dissolution mechanisms caused by the presence of HCl.

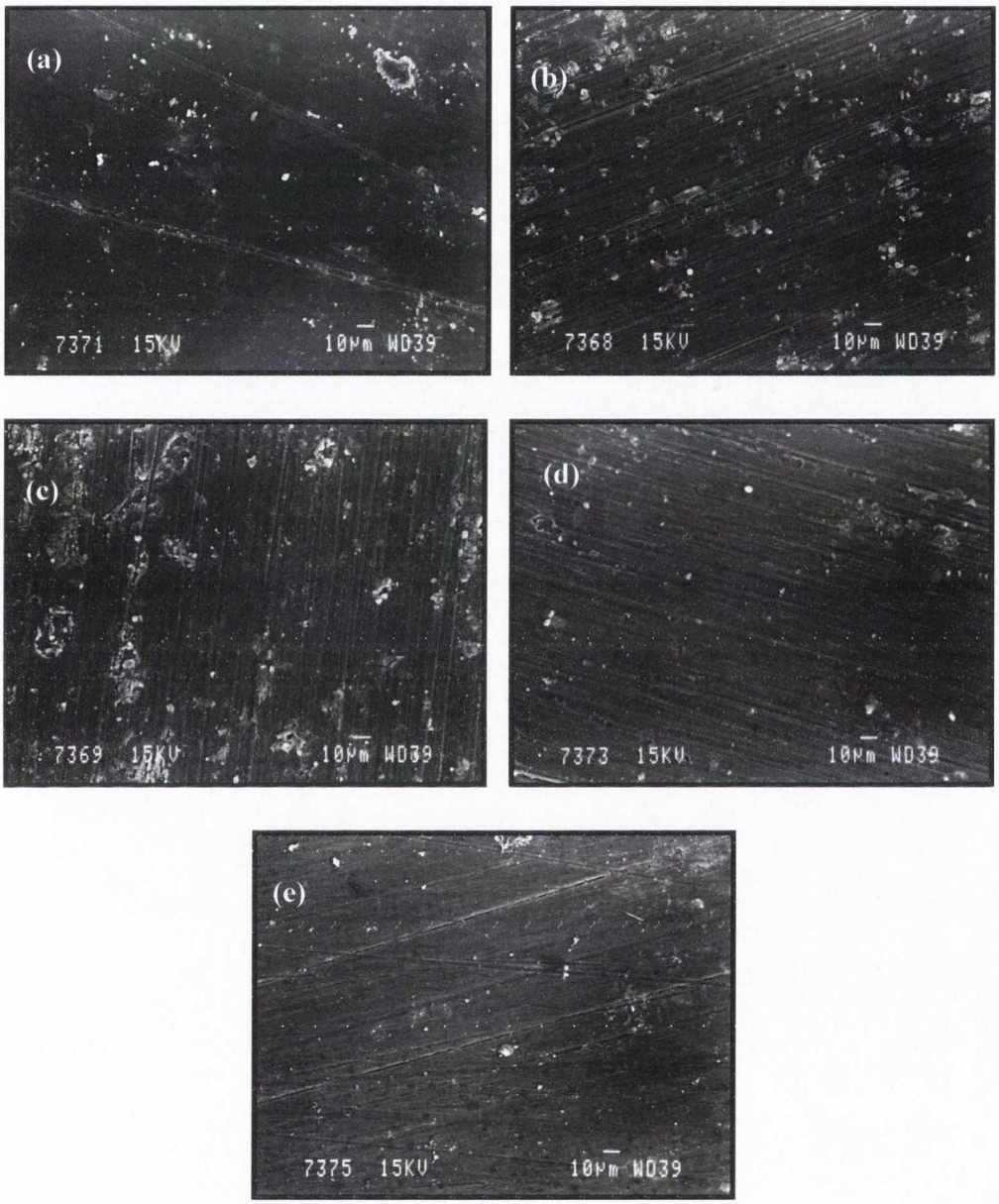

Figure 5.2: Scanning electron microscopy of β-TCP discs before and after incubation (Day 12) with various HCl concentrations. (a) β-TCP disc before incubation (b) 0mM HCl incubated at 10% CO_2 (c) 0mM HCl incubated at 5% CO_2 (d) 10mM HCl incubated at 5% CO_2 (e) 15mM HCl incubated at 5% CO_2 Magnification x450, scale bar 10µm.

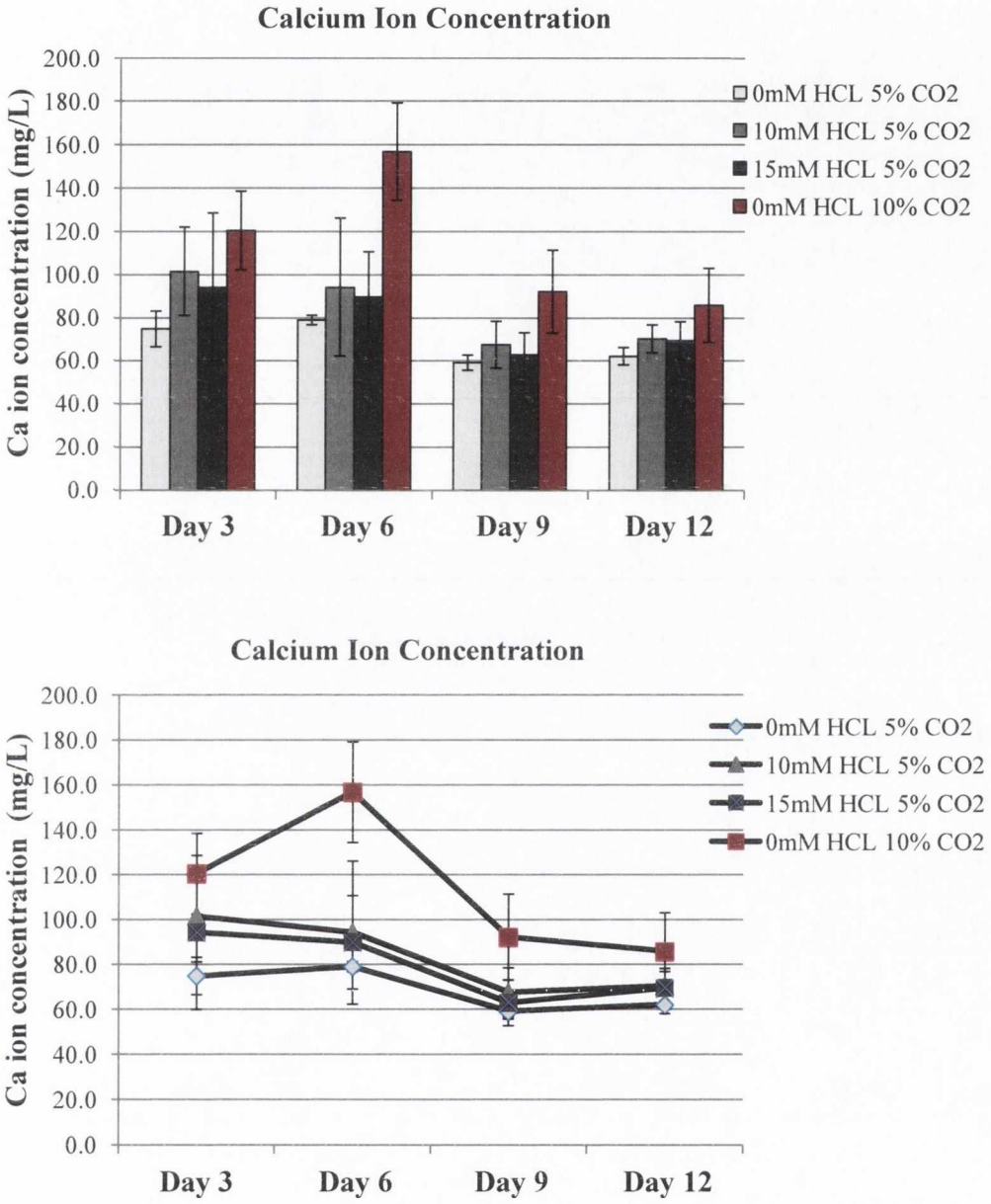

Figure 5.3: Bar chart and line graph of calcium ion concentration in culture medium incubated with various concentrations of HCl or percentage CO_2.

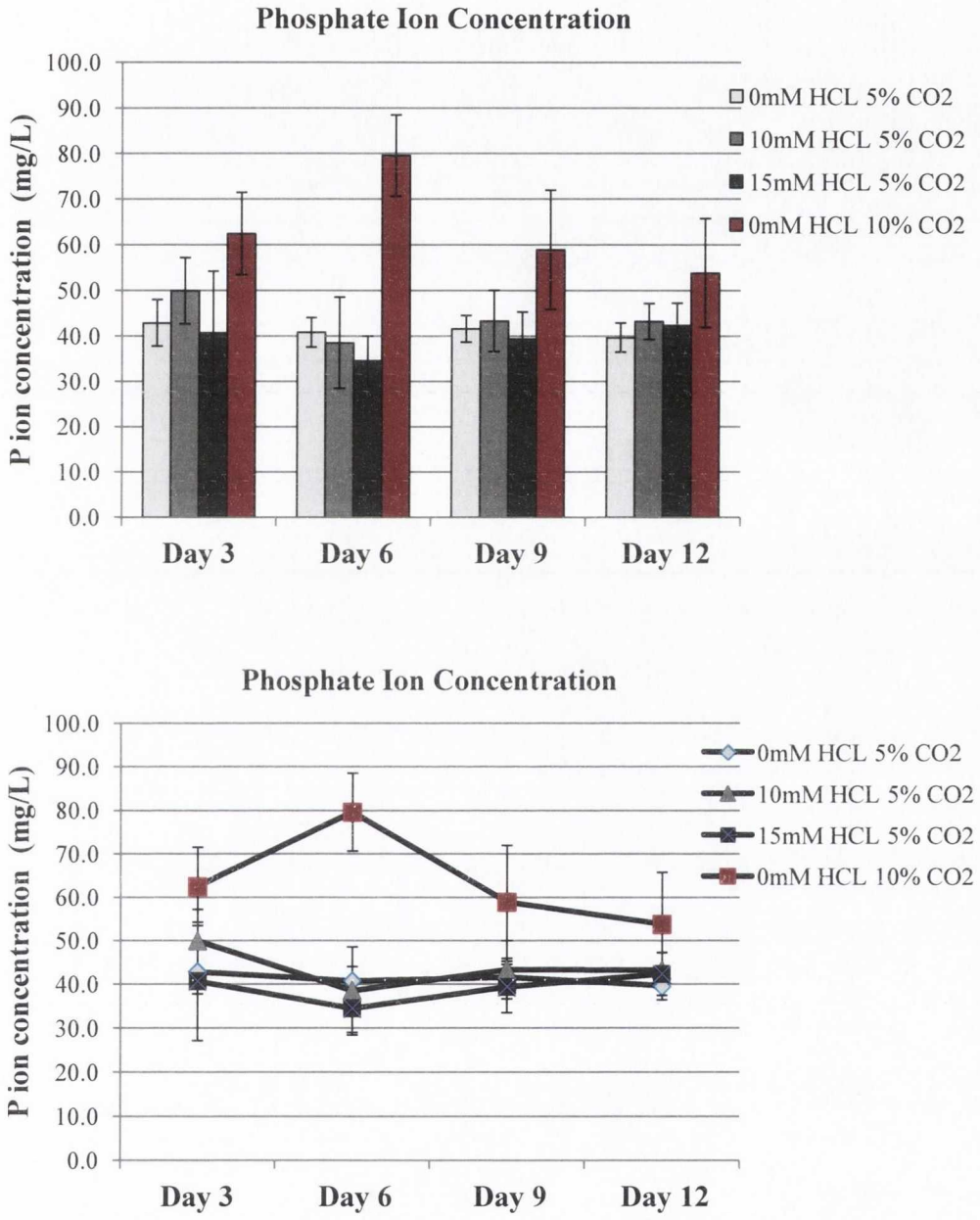

Figure 5.4: Bar chart and line graph of phosphate ion concentration in culture medium incubated with various concentrations of HCl or percentage CO_2.

5.4 Experiment E

A further cell free investigation was conducted to assess the effects of HCl addition on the pH of the culture medium with time. The aim of this experiment was to investigate if HCl addition to the culture medium brings culture medium pH close to the optimal range of pH 6.95 for increasing OC resorptive activity [41].

5.4.1 Materials and Methods

Preparation of β-TCP discs

Dense β-TCP cylinders were produced as described in Section 4.2.4.1. The cylinders were then cut into 4mm thick discs using a diamond wire saw (Struers Accutom-50). Each slice was flattened by manually grinding with silicon carbide paper grit P500 (grain size 30μm) (Struers). The following grinding and polishing cycle was then carried out on one surface of the disc:

- SiC P400 – 3 minutes or until planar
- SiC P1200 – 3 minutes
- SiC P2500 – 3 minutes
- SiC P4000 – 3 minutes
- Final polish on Masterpolish cloth – 3 minutes (All consumables Beuhler)

Discs were then washed with 70% isopropyl alcohol (Sigma Aldrich) and sterilised by autoclaving at 121°C for 30 minutes.

15 discs were placed into 96-well plates and covered in 200μl of α-MEM, supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen) and 10% (v/v) FBS,

(Sigma-Aldrich) and incubated under standard conditions (37°C, 5% CO_2/95% air). A separate group of samples (N=5) were incubated at 37°C, 10% CO_2/90% air. All samples were incubated for 12 days. Media was replaced every three days. Of the 15 samples incubated under standard conditions 5 of these received 10mM HCl and 5 received 15mM HCl when media was replaced on Days 3, 6 and 9. The remaining 5 samples received no HCl. The overall group names to be tested were as previous: 0mM HCl 5% CO^2, 10mM HCl 5% CO^2, 15mM HCl 5% CO^2 and 0mM HCl 10% CO^2.

pH

pH levels were recorded using a Hanna Micro refillable pH electrode and a Hanna pH Meter (Analab,UK). Culture media was analysed daily (N=5). 10µl of culture medium was placed into a 1.5ml Eppendorf vial and the lid of the vial was sealed immediately. Sample sets that were not being analysed immediately were placed into the respective incubator. The pH electrode was rinsed with dH_2O between readings.

5.4.2 Results

The Initial pH levels from Day 0 to Day 3 highlight the effect of increasing the percentage CO_2 in the absence of HCl. An increase in percentage CO_2 to 10% increased the acidity of the culture medium. At Day 0 the culture medium had a pH level of 8.04 and after one day incubation the pH level for culture medium at 5% CO_2 was ~ pH 8.25 whereas the pH level at 10% CO_2 was ~pH 7.65 (Figure 5.5). This indicates that increasing the % CO_2 has an acidifying effect on culture medium. When CO_2 reacts with water (H_2O) carbonic acid (H_2CO_3) is formed and can

subsequently break up into hydrogen (H^+) and bicarbonate ions (HCO_3^-). It has been shown that increasing the % CO_2 allows acidification of culture medium whilst HCO_3^- remains constant [133].

After addition of acid at Day 3 there is a significant reduction in pH for 10mM and 15mM HCl groups at 5% CO_2, as expected. Direct addition of HCl to culture medium has been shown to reduce pH by reducing HCO_3^- [133]. The 0mM HCl 10% CO_2 group also had a significant reduction in pH after Day 3 whereas the 0mM HCl 5% CO_2 group remained relatively constant. All groups become more alkaline with time between culture medium changes (Days 3, 6 and 9). There is an initial reduction in pH after medium change followed by a stabilisation phase.

After a change in culture medium at Day 6 the 15mM HCl 5% CO_2 group becomes the most acidic group for the remainder of the study. Ranging from more alkaline to more acidic the trend after Day 6 is as follows: 0mM HCl 5% CO_2, 10mM HCl 5% CO_2, 0mM HCl 10% CO_2 and 15mM HCl 5% CO_2. This shows that after Day 6 the addition of 15mM HCl to culture medium at 5% CO_2 has the potential to acidify culture medium to a greater extent than increasing the percentage CO_2. The 15mM HCl 5% CO_2 group had the most acidic pH level at Day 10 (pH 7.36) and was the closest to the optimal pH 6.95 reported for resorption pit formation [41].

This experiment was carried out in a cell free environment. Metabolic activity of cells is acidic in nature and further reduces culture medium pH. With the optimal pH for OC formation between pH 7.35-7.4 [134] the pH results of this experiment indicate that 15mM HCl added to culture medium at 5% CO_2 may provide sufficient acidification to firstly allow OC formation at a higher pH (pH ~7.4) followed by optimisation of activity and resorption pit formation at a lower pH with time.

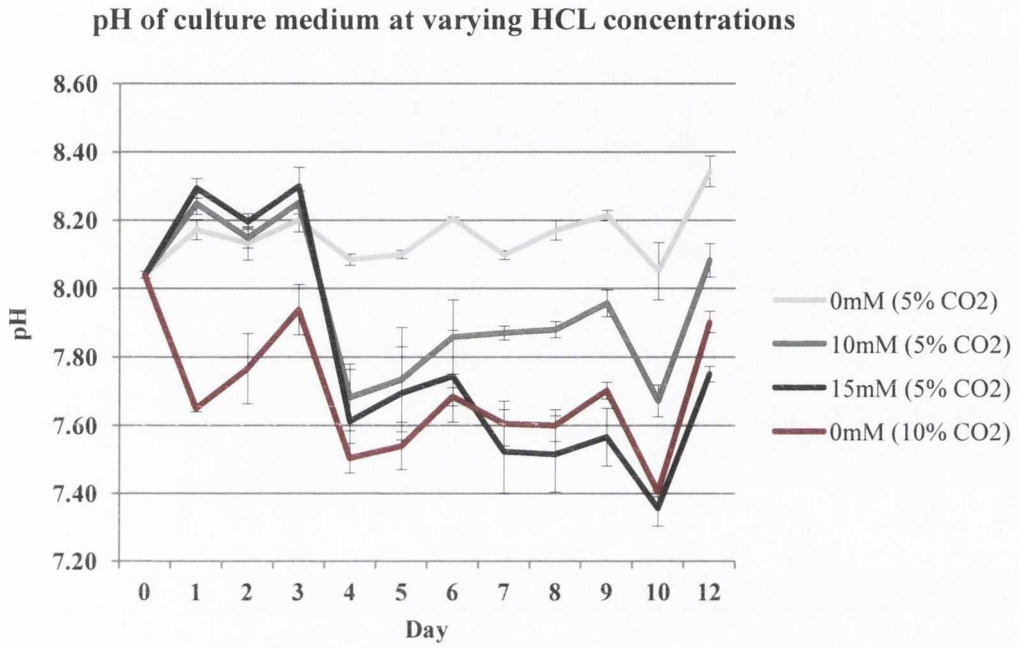

Figure 5.5: pH of culture medium incubated with various concentrations of HCl or percentage CO_2.

5.5 Overall Conclusions

After assessing the effects of adding 15mM HCl to culture medium the following conclusions were drawn:

- There was no difference in the surface of the β-TCP disc before and after incubation with or without HCl addition to cell culture medium.
- 15mM HCl addition produced calcium and phosphate ion concentrations close to 0mM HCl at 5% CO_2 and therefore did not impact the normal functioning of the β-TCP disc in culture medium.
- The resorption pits obtained in Switzerland using 15mM HCl at 5% CO_2 were cell-mediated rather than by dissolution mechanisms caused by the presence of HCl.
- Addition of 15mM HCl to culture medium at 5% CO_2 in a cell free environment acidified the culture medium to a near optimal level for OC formation after Day 6.
- Further acidification of culture medium is expected with the inclusion of cells to culture medium, due to cellular metabolic activity. Further acidification is desired when trying to increase resorption pit formation by OC.

Considering the above conclusions, 15mM HCl will be added to culture media as a permanent modification of the initial protocol to ensure acidification of the culture medium for the optimisation of OC formation and resorption.

The final modifications to the OC assay are as follows:

- 20ng/mL RANKL (PeproTech) will be added to culture medium for the differentiation of OC.
- 15mM HCl will be added to culture medium from Day 3 to optimise OC formation and activity.

The optimised OC assay will be used to generate RAW 264.7 OC for the resorption of β-TCP for the assessment of various outcome measures used to quantify resorption *in vitro*.

CHAPTER SIX

Surrogate outcome measures as indicators of *in vitro* osteoclast resorption of calcium phosphate ceramics

6 Surrogate outcome measures of *in vitro* osteoclast resorption of calcium phosphate ceramics

6.1 Introduction

The traditional *in vitro* method used to assess resorbability of bone substitutes is a cell-based resorption assay, alternatively known as a 'pit' assay, developed by Boyde [57] and Chambers [40]. The initial assay was developed using dentine and bone respectively [57][40], but is now routinely used to understand biomaterial resorption. OCs are cultured on the bone substitute surfaces for specific periods and then detached, at which point the excavated areas (pits) beneath the cells can be analysed by SEM.

There are three fundamental options used when quantifying resorption; pit number, pit area and pit volume. The simplest method is to determine the number of pits, which can be quantified using reflected light microscopy (RLM), where staining is not required [135] or by light microscopy (LM) using simple staining techniques, such as toluidine blue and acid haematoxylin [67]. Pit area can be quantified using image analysis software applied to SEM or LM. These methods are very time consuming, labour intensive, and do not easily translate to quantification of resorption on porous materials where visualisation of internal structures is difficult. Ideally, pit volume would be the best method when quantifying resorption as both pit area and depth can be calculated. However, the required equipment is both expensive and specialised [67][136][137][138][139]. Thus, there is an imperative for an appropriate measure of resorption that is efficient and more cost-effective than the current available *in vitro* methods, which can be adopted for both porous and dense calcium phosphate ceramics.

Other methods used to indicate OC resorption are based on their activity, generally assessed using biochemical markers such as the OC specific enzyme, tartrate resistant acid phosphatase (TRAP). TRAP activity is commonly measured using either a colorimetric method [127][140] or by using an enzyme-linked immunosorbent assay (ELISA) which uses TRAP specific antigen-antibody reactions to measure TRAP activity [141][142][143][144], also measured by absorbance. Another commonly used *in vitro* biochemical assay is a colorimetric calcium assay [64][95] .

Many of the methods listed above are suitable as indicators of OC resorption on the basis of biomaterial biocompatibility, identifying possible mechanisms that modulate the rate of resorption or for the investigation of drug-OC interactions and their effect on resorption. However, there has been no systematic attempt to identify an outcome measure of OC resorption that directly correlates with pit measurements and is transferrable through a broad range of *in vitro* experiments.

The aim of this study was to establish the suitability of several outcome measures as possible indicators of osteoclastic resorption *in vitro*. This study investigated the correlation of Ca and P mineral release into cell culture medium with the percentage area resorbed *in vitro*, OC number and activity. In order to accurately correlate pit area with the alternative outcome measures the assay was carried out using dense β-TCP. The rationale for using a dense substrate was two-fold. Firstly, it allowed pit formation and area to be analysed devoid of material anomalies or pores, which are frequently observed with less dense substrates and can give rise to discrepancies within results. Secondly, the current 2D methods for measuring resorption perform best on a substrate free from imperfections.

6.2 Materials and Methods

6.2.1 Preparation of ceramic discs

Dense β-TCP cylinders were produced in accordance with Section 4.2.4.1. The final cylindrical samples (diameter 3.8 mm x 30 mm) were cut into 3 mm thick discs using a diamond saw (Struers Accutom-50). Each disc was mounted in acrylic resin (Varidur 3000, Buehler) and ground (Buehler Alpha Grinder-Polisher) on one side using silicon carbide papers of decreasing grade (P400, P1200, P2500, P4000) followed by a final polish using a 0.05μm alumina and silicon oxide suspension (Buehler). Each grinding and polishing stage was conducted for 3 minutes. Polished discs were removed from the acrylic resin using a 48 hour soak in chloroform, then washed with 70% isopropyl alcohol (Sigma Aldrich) and sterilised by autoclaving at 121°C for 30 minutes.

6.2.2 Cell culture of RAW 264.7 cells

RAW 264.7 cells (ATCC) were routinely cultured under standard conditions (37°C, 5% CO_2/95% air) in α-MEM medium, supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin and 4mM L-glutamine (all reagents Invitrogen). At day 0, cells were seeded onto the polished side of β-TCP discs at a density of $2.5x10^4$ cells/cm^2. To initiate differentiation, RANKL (PeproTech) was filter sterilised (0.2μm filter) and added to complete culture medium at a final concentration of 20ng/mL. Culture medium and RANKL was replaced every 3 days. 15mM HCl (Sigma Aldrich) was added to cultures on days 3, 6 and 9. Cultures were maintained for 12 days. N=6 for all conditions.

6.2.3 Actin staining of cells

At day 6, 8, 10 and 12, cultures were fixed and stained with AlexaFluor 488 Phalloidin and DAPI dilactate (Invitrogen) in accordance with Section 4.2.4.4. Cultures were imaged under fluorescence microscopy (Leitz-Laborlux D) at x16 magnification. The surface area (SA) analysed per field of view was $1mm^2$. Based on four fields (SA $4mm^2$), 35% of a 3.8mm disc (SA $11.34mm^2$) was analysed. Actin ring number was calculated from the mean of the fields (N=6). Multiple actin rings within an OC were counted individually with an osteoclast defined as having three or more nuclei.

6.2.4 TRAP staining and activity

OC differentiation was determined using a TRAP staining kit (Sigma-Aldrich). On day 6, 8, 10 and 12, cultures were fixed in citrate/acetone solution for 30 seconds, washed in dH_20 and air dried for 15 minutes. Cultures were then covered in a solution containing napthol AS-BI phosphoric acid and fast garnet GBC salt and incubated for 1 hour at 37°C in the dark. Cultures were rinsed with dH_2O for 3 minutes and allowed to air dry before viewing under LM (Leitz-Laborlux D) at x16 magnification. The method for counting the mean number of TRAP positive OC cells was similar to the actin ring count (Section 6.2.3). Using four fields of view, 35% of a 3.8mm disc was analysed.

OC TRAP activity was carried out in accordance with Section 4.2.1.5 for Days 6, 8, 10 and 12 (N=6). Optical absorbance was read at 405nm on a microplate reader (Genios, Tecan, Austria) and TRAP activity was quantified against a standard curve (p-NP).

6.2.5 pH

pH levels were recorded using a Hanna Micro refillable pH electrode and a Hanna pH Meter (Analab,UK). Culture medium was analysed daily for culture medium containing cells (N=6) and on Days 0, 3, 6, 9 and 12 for cell free culture medium (N=6). 10µl of culture medium was placed into a 1.5ml Eppendorf vial and the lid of the vial was sealed immediately. Sample sets that were not being analysed immediately were placed into the respective incubator. The pH electrode was rinsed with dH_2O between readings.

6.2.6 Elemental analysis

Elemental concentrations of Ca and P ions were quantified using ICP-MS (Perkin Elmer Optical Emission Spectrometer, Optima 4300DV). Culture media from days 3, 6, 9 and 12 were retained and diluted by a factor of 80 in dH_2O (0.05ml sample to 3.95ml dH_2O) for analysis (N=6).

6.2.7 SEM of resorption pit formation by osteoclasts

Day 6, 8, 10 and 12 cultures on discs were washed and fixed in accordance with Section 3.2.3. The dried specimens were then sputter coated with gold using a Polaron E5150 sputter coater and viewed on a Jeol SEM at 15kV (N=6).

6.2.8 Quantification of resorption pit area

Resorption pit area measurements were performed using Image J v1.45 software (National Institute of Health, Bethesda, USA). A threshold function was used to convert the SEM image into binary mode followed by a particle analysis function to quantify the percentage area resorbed by the RAW 264.7 OC cells. At x100 magnification (SA 1.13mm^2/field of view), 30% of a 3.8mm disc was analysed. The

percentage area resorbed was calculated from the mean of the fields (3 fields/sample, N=6). A resorption pit was defined as an excavated area >30 μm in diameter.

6.2.9 Statistics

Statistical Analysis for all outcome measures was carried out using SPSS v.18 software (IBM, USA). Kolmogorov-Smirnov and Shapiro-Wilk normality tests were conducted and all groups were normally distributed except for the percentage area resorbed group (Area). Logarithm transformation of Area (LogArea) data was normally distributed. Differences between treatment groups were assessed using ANOVA with a post-hoc, Bonferroni test. Relationships between each outcome measure were investigated using the Pearson's correlation test. A p-value of less than 0.05 was considered statistically significant. A total of six samples were used per time point for all conditions.

6.3 Results

6.3.1 TRAP activity and TRAP positive osteoclast number

TRAP activity increased from Day 6 (AU 0.61) to Day 10 (AU 1.02) and decreased at Day 12. TRAP activity for cells/+RANKL was the same at Days 8 and 12 with a mean absorbance of 0.87 AU (

Figure **6.1**). Cells/-RANKL control samples displayed similar absorbance readings to cells/+RANKL samples at Days 4 and 12 and TRAP activity for Day 12 cells/-RANKL (monocytes only) was higher than Day 6 cells/+RANKL (OC present) indicating that RAW 264.7 monocytes produce a basal level of TRAP enzyme regardless of RANKL stimulation and thus TRAP activity alone is not a reliable

quantitative outcome measure for OC activity. TRAP activity is however a useful indicator for OC number.

Formation of TRAP positive OC occurred from Day 6. The mean number of TRAP positive OC increased from Day 6 (9 OC) to Day 10 (28 OC) and decreased at Day 12 (17 OC) (Figure 6.2). TRAP positive OC results support the trends observed for TRAP activity. The largest change in OC number was from Day 8 to Day 10 increasing by 87% (15 OC to 28 OC respectively), followed by a 40% decrease from Day 10 to Day 12.

6.3.2 Actin ring formation and number

Actin ring formation did not follow the same trends observed with TRAP positive OC count and TRAP activity results. Large standard deviations were observed for all timepoints (Figure 6.2). Fluorescence microscopy (Figure 6.3) indicated that the size and shape of actin rings formed changed with time. At the earlier timepoint of Day 6 there were smaller actin rings than Day 10 or 12. Day 8 displayed a range of actin ring size from small to large. Most Day 10 and Day 12 actin rings were large and covered a lot of the sample surface compared to the earlier timepoints. This variation in actin ring size may explain such large standard deviations.

Both Day 6 and Day 10 had the same mean number of actin rings (mean actin ring number 11, n=6) (Figure 6.2). Thus, actin ring number as a function of mean actin rings formed can be misleading. Results displayed as a ratio of the mean number of actin rings to TRAP positive OC show a distinct decreasing trend in the ratio of actin rings to TRAP positive OC with time (Figure 6.4). Standard deviation remains high however there is a 3 fold decrease in the ratio of actin rings to TRAP positive OC from Day 6 to Day 10.

6.3.3 pH of culture medium

pH values of culture medium were similar for cells +/- RANKL (Figure 6.5) with a general decrease in pH up to Day 8 followed by a slight increase until Day 12. Days 5 and 6 exhibited the most difference in pH between cells +RANKL and cells –RANKL with cells +RANKL having a decreased pH. At Day 8, cells +RANKL and cells –RANKL displayed near exact pH levels, pH 6.87 and pH 6.85 respectively.

After addition of 15mM HCl to culture medium at Day 3, a marked difference in pH can be observed between cell cultures and cell free cultures (Figure 6.5). Cell free culture medium decreased by an overall pH difference of 1.0 from Day 3 to 12 (pH 8.44 to 7.44 respectively). Cells +RANKL and cells –RANKL culture medium decreased by an overall pH difference of 1.03 and 0.83 respectively. The overall pH change in each group was similar (~1.0 pH unit) however, cell free culture medium pH remained at a higher level (more alkaline) throughout. Cell free culture medium pH continued to decrease throughout whereas culture medium containing cells had a decrease throughout followed by a slight increase from Days 9 to 12.

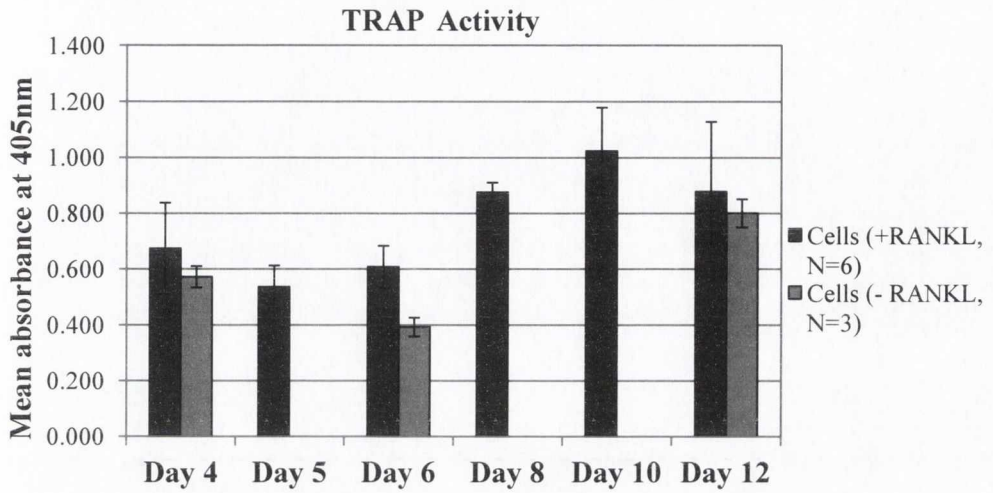

Figure 6.1: TRAP activity of RAW 264.7 cells on β-TCP after 12 days.

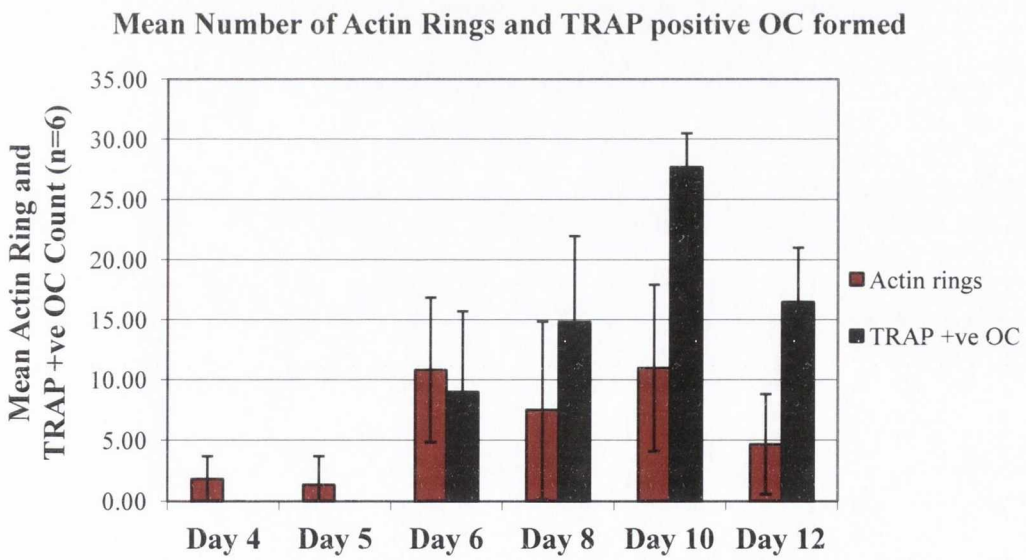

Figure 6.2: Mean Number of Actin Rings and TRAP positive Osteoclasts formed.

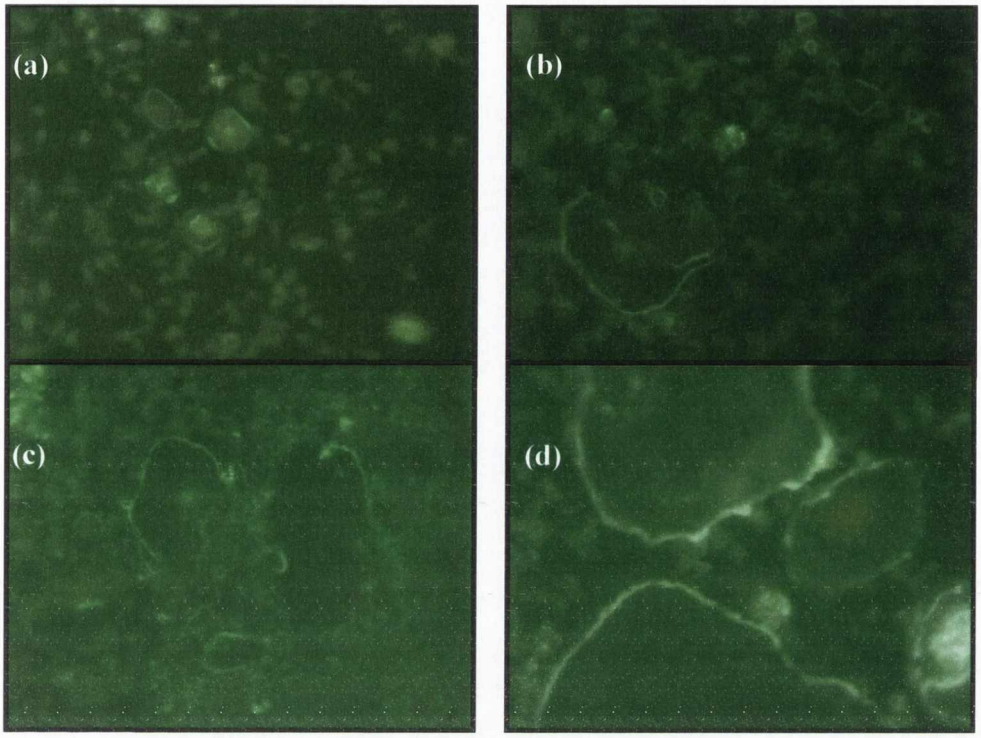

Figure 6.3: Fluorescence microscopy of RAW 264.7 OC actin rings. x16 magnification., (a)- (d) Days 6, 8, 10, 12 respectively.

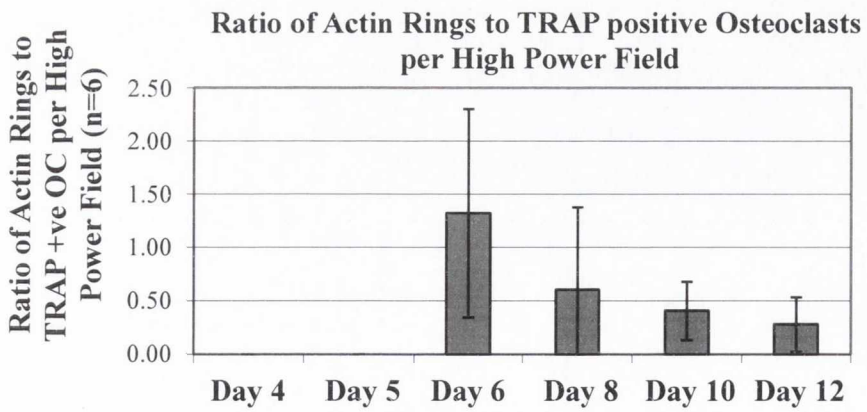

Figure 6.4: Ratio of Actin rings to TRAP positive OC per high Power Field.

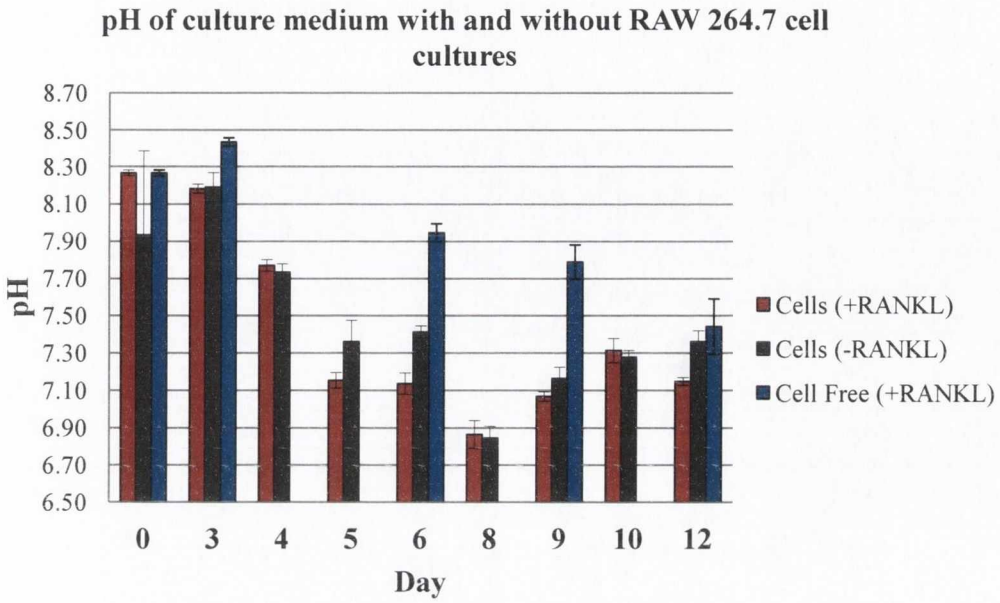

Figure 6.5: pH of culture medium with and without RAW 264.7 cells.

6.3.4 Element concentrations in culture medium

Mineral ion release into culture medium was time dependent and reflected the trends observed with TRAP activity and OC number. Ca ion concentration increased from Days 3 to 9 and a decreased at Day 12 whereas P ion concentration remained relatively stable from Days 3 to 9 and decreased at Day 12 (Figure 6.6).

Basal Ca ion concentration in culture medium was 40mg/L. There was a significant increase in Ca ion concentration with time with maximum Ca ion concentration of 66.8mg/L at Day 9. Days 3 and 9 exhibited a highly significant difference in Ca ion concentration in culture medium with cells compared to culture medium without cells (***$p<0.001$). Basal P ion concentration in culture medium was 25mg/L. Maximum P ion concentration occurred at Day 9 (36.5mg/L). Significant differences

were also observed for culture medium with cells compared to culture medium without cells at Days 9 and 12 (** $p<0.01$).

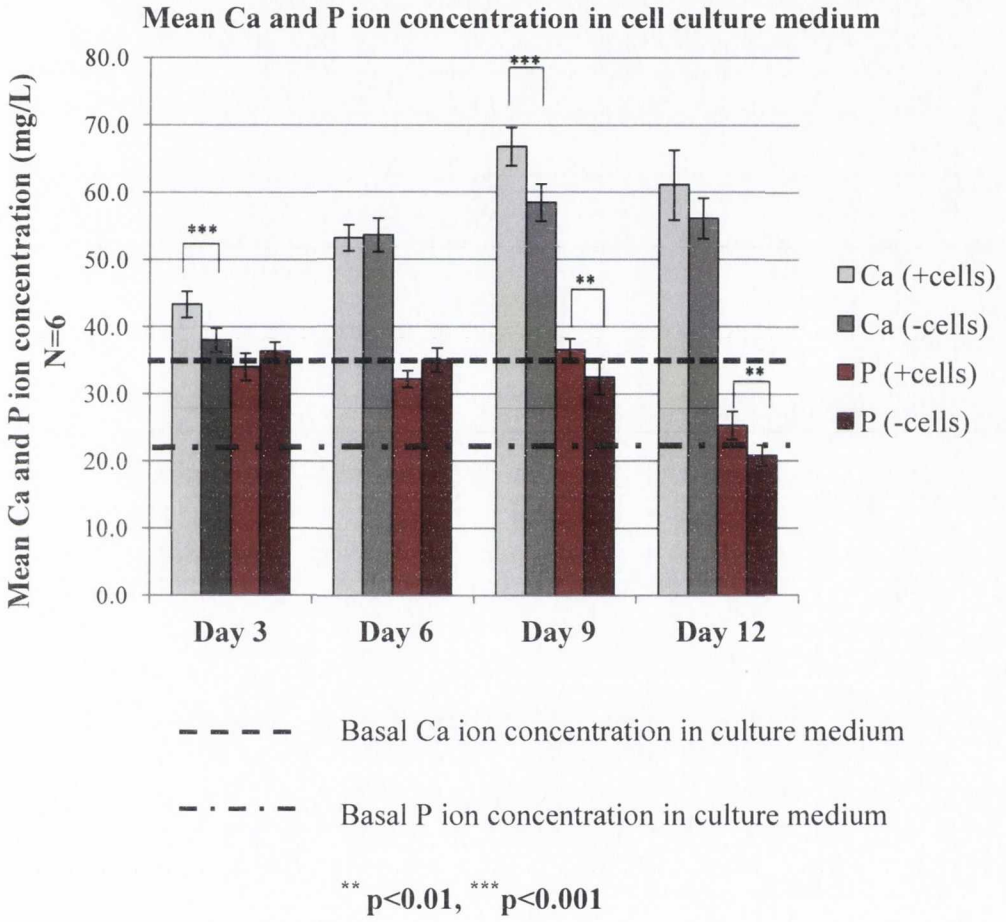

Figure 6.6:Mean Ca and P ion concentration in cell culture medium after 12 days. Basal Ca ion concentration in culture medium was 40mg/L. Basal P ion concentration in culture medium was 25mg/L.

6.3.5 Resorption pit formation and quantification of resorption

Light coloured areas on SEM micrographs represent resorption pits (Figure 6.7). Resorption pits formed were circular or lobe shape and were not observed until Day 8. Resorption significantly increased at Day 8 (Figure 6.8). At x100 magnification, 30% of the total surface of the β-TCP sample was analysed. Maximum surface resorption recorded was ~20%. There was no significant difference in percentage area resorbed between Days 8 and 12 and standard deviation was large within each timepoint (Figure 6.8). Higher magnification SEM micrographs illustrate that resorption pits appear deeper with time (Figure 6.7).

6.3.6 Statistical analysis

Statistical analysis showed all outcome measures to have a highly significant difference when assessed as a function of time. A One way ANOVA was carried out on all outcome measures and the relationship between each outcome measure was correlated (Table 6.1).

Results for TRAP activity and TRAP positive OC count showed good correlation with each other (r = 0.670**). OC count also showed good correlation with Ca ion concentration (r = 0.708**). Ca ion concentration showed good correlation with pH (r=-0.740**). Log area had a moderate correlation with TRAP activity, OC count and Ca ion concentration (r = 0.447*, 0.450* and 0.599 respectively). Negative correlations were observed between pH and Ca ion concentration (r = -0.740**), pH and OC count (r = -0.420**) and pH and actin ring coun (r = -0.359*).

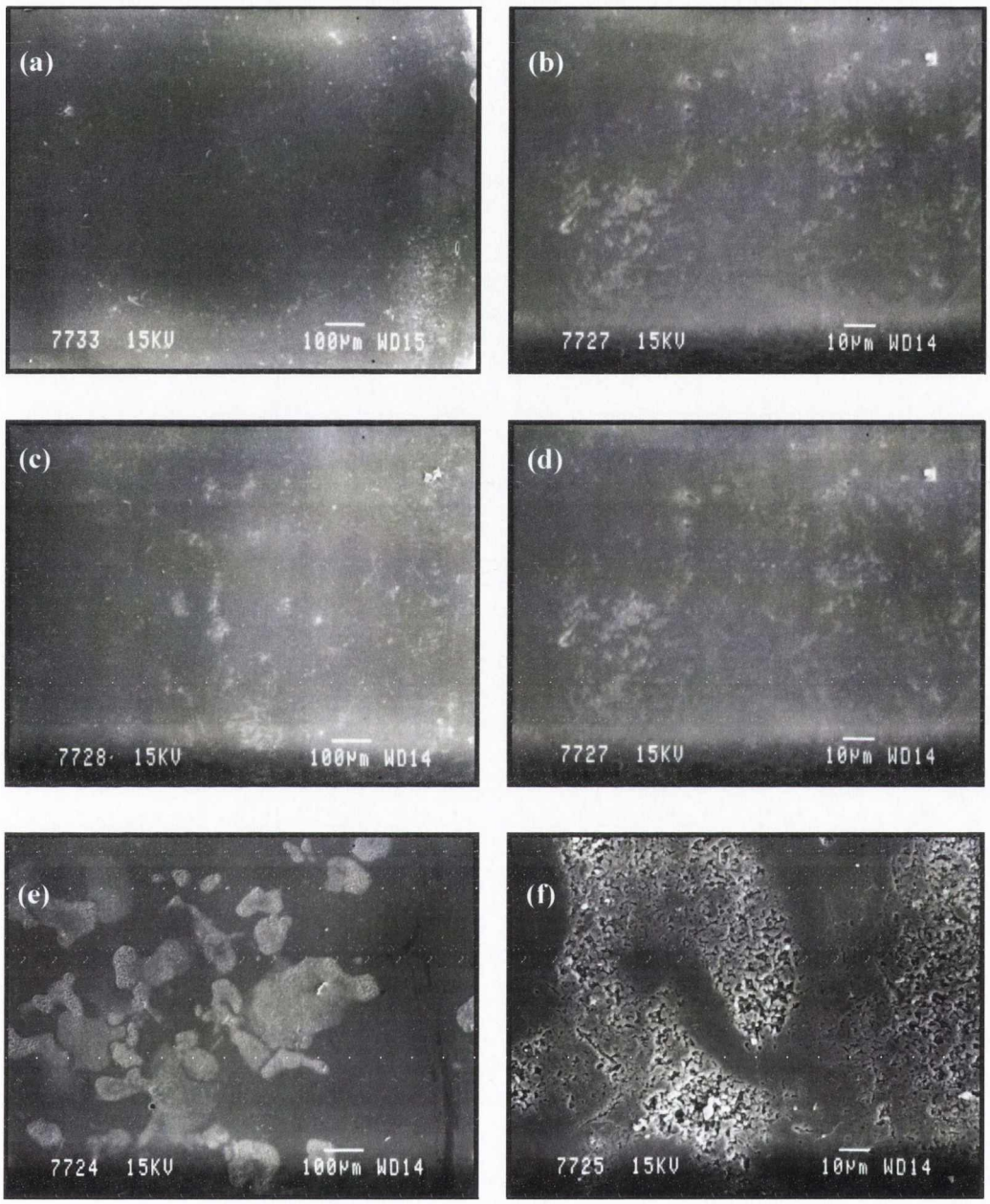

Figure 6.7: SEM micrographs of β-TCP resorption by RAW 264.7 OC after 12 days. (a-b) Day 4, (c-d) Day 6, (e-f) Day 8, (g-h) Day 10 (i-j) Day 12. (a c, e, g, i) x100 magnification, scale bar 100µm, (b, d, f, h, j) x800 magnification, scale bar 10µm.

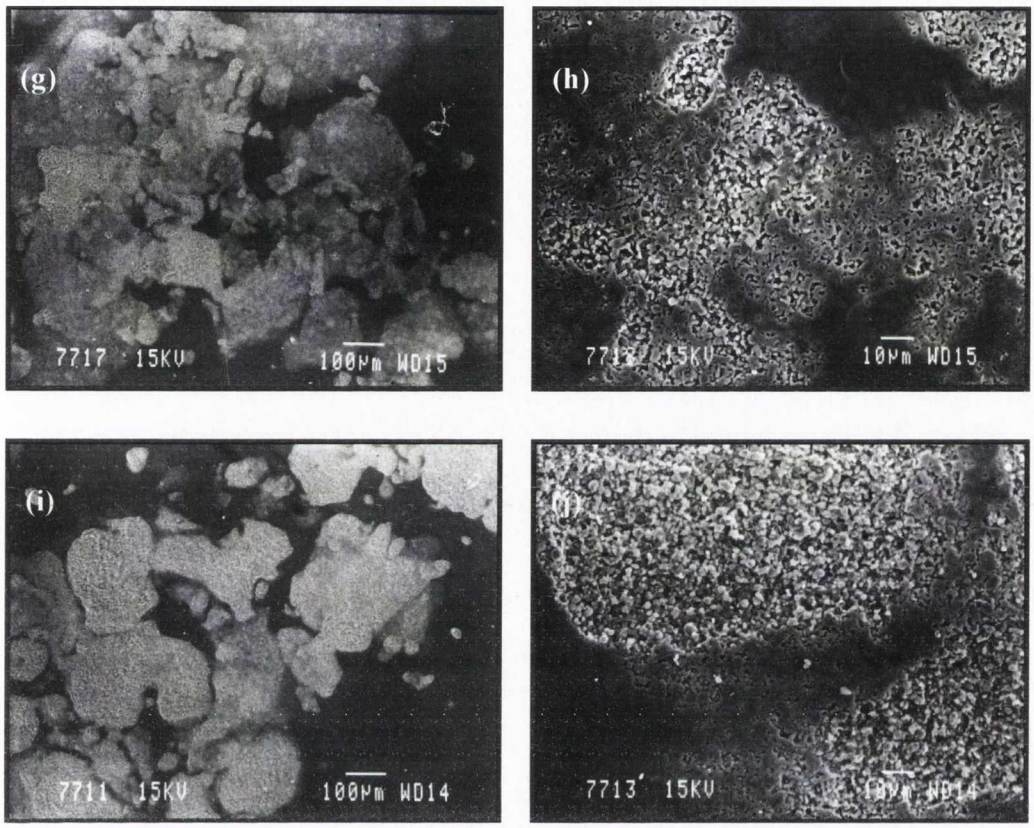

Figure 6.7 continued: SEM micrographs of β-TCP resorption by RAW 264.7 OC after 12 days. (a-b) Day 4, (c-d) Day 6, (e-f) Day 8, (g-h) Day 10 (i-j) Day 12. (a c, e, g, i) x100 magnification, scale bar 100µm, (b, d, f, h, j) x800 magnification, scale bar 10µm.

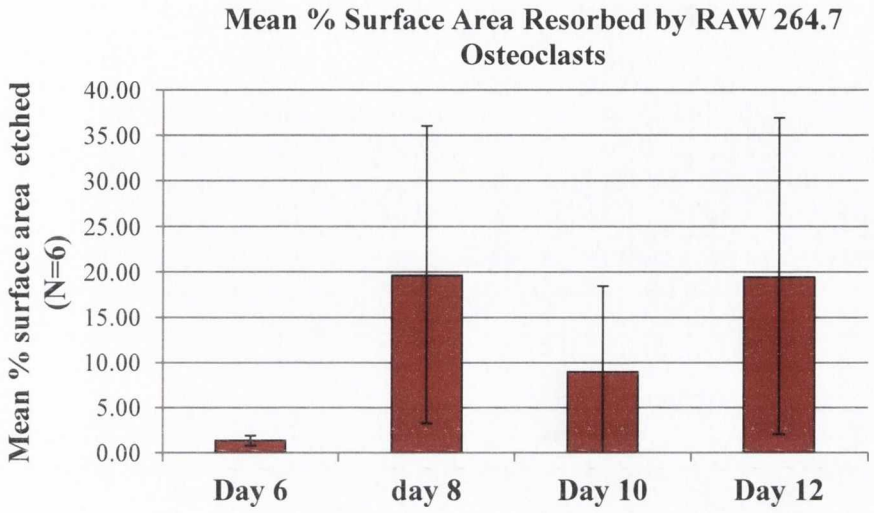

Figure 6.8: Percentage surface area resorbed by RAW 264.7 OC after 12 days.

Table 6.1: Correlation table representing statistical analysis carried out on all outcome measures. The top figure in each row represents the Pearson correlation factor (r) showing the strength of correlation and the bottom figure indicates the significance of the results (p-value). TRAP = TRAP activity, AR = Actin ring count, OC = TRAP positive OC count, Ca = Ca ion concentration, P = P ion concentration, AR:OC = Ratio of actin ring to TRAP positive OC, logArea = log of the percentage area resorbed. **Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level.

	TRAP	pH	AR	OC	Ca	P	AR:OC	logArea
TRAP			0.381**	0.670**	0.572*	-0.775**		0.447*
			0.010	<0.001	0.013	<0.001		0.037
pH			-0.359*	-0.420**	-0.740**	0.385**		
			0.015	0.003	<0.001	<0.001		
AR	0.381**	-0.359*		0.553**			0.625**	
	0.010	0.015		<0.001			<0.001	
OC	0.670**	-0.420**	0.553**		0.708**		-0.492*	0.450*
	<0.001	0.003	<0.001		<0.001		0.017	0.036
Ca	0.572*	-0.740**		0.708**				0.599
	0.013	<0.001		<0.001				0.510
P	-0.775**	0.385**						-0.783**
	<0.001	<0.001						0.004
AR:OC			0.625**	-0.492*				-0.439*
			<0.001	0.017				0.046
Log Area	0.447*			0.450*		-0.783**	-0.439*	
	0.037			0.036		0.004	0.046	

6.4 Discussion

The aim of this study was to identify an outcome measure(s) of OC resorption that directly correlates with OC pit measurements and is transferrable through a broad range of *in vitro* experiments. This study investigated the correlation of Ca and P mineral release into cell culture medium with the percentage area resorbed *in vitro*, OC number and activity. In order to accurately correlate pit area with the alternative outcome measures the assay was carried out using dense β-TCP.

OC TRAP activity results increased from Day 6 to Day 10 and decreased thereafter. Cells -RANKL control samples displayed similar absorbance readings to cells +RANKL samples at Days 4 and 12 and TRAP activity for Day 12 cells/-RANKL (monocytes only) was higher than Day 6 cells/+RANKL (OC present) indicating that RAW 264.7 monocytes produce a basal level of TRAP enzyme regardless of RANKL stimulation and thus TRAP activity alone is not a reliable quantitative outcome measure for OC activity.

Active OC express higher levels of TRAP activity compared to inactive OC [145]. A possible explanation why Day 12 cells +RANKL (0.87 AU) were close to Day 12 cells -RANKL (0.8AU) could be that Day 12 cells -RANKL samples were covered in a confluent monolayer of monocytes or mononuclear prefusion OC that express TRAP activity [56] and Day 12 cells +RANKL samples previously contained mature active OC of which were no longer active. Thus, TRAP activity levels for cells +RANKL decreased to a similar level as cells -RANKL due to the level of confluency (i.e. individual monocytes express less TRAP activity compared to active OC however the level of TRAP activity observed is a cumulative effect due to the number of monocytes present relative to the number of OC).

OC have been shown to secrete more TRAP *in vitro* than *in vivo*, suggesting that TRAP is secreted more readily *in vitro* but may be regulated more efficiently in a

receptor-mediated microenvironment *in vivo* [127]. To back up any trends in TRAP activity a TRAP positive OC count was performed where an OC was denoted as having 3 or more nuclei. TRAP positive OC results supported the trends observed for TRAP activity with an increase in OC number from Day 6 to Day 10 followed by a 40% decrease from Day 10 to Day 12. Results for TRAP activity and TRAP positive OC count showed good correlation with each other ($r = 0.670^{**}$) indicating that TRAP activity is an indicator of OC number.

When an OC is polarised on a substrate surface a sealing zone forms containing a dense ring of actin. The presence of the actin ring is indicative of the resorptive phase of the OC [4][31][36]. This study aimed to assess the suitability of actin ring formation as an indicator of OC resorption. Actin ring number was assessed. Actin ring formation did not follow the same trends observed with TRAP positive OC count and TRAP activity results. Large standard deviations were observed for all timepoints. One may expect to see an increase in actin ring formation with an increase in OC number however this was not the case. Observations from fluorescence microscopy indicated that the size and shape of actin rings formed changed with time. This variation in actin ring size may explain such large standard deviations. In general, actin ring size increased with time. This resulted in a decreasing trend in the ratio of actin rings to TRAP positive OC with time. An alternative method may be to record the diameter of individual actin rings rather than counting actin rings. This may correlate better with OC resorption activity where pit area measurements are used to determine activity.

The pH of culture medium was assessed as a means of looking at the conditions of the culture medium with time and to aid interpretation of any resorption trends observed. pH values are similar for both cells +/- RANKL groups. Overall environmental pH within a single well is buffered by cells and the CO_2 environment within the incubator [133]. OC exhibit a change in pH when they are in their active

state but this change is within a localised region beneath the cell delineated by the sealing zone and ruffled border [46][47][45]. Therefore cells +RANKL groups did not generally exhibit an overall significant decrease in pH (increase in acidity) compared to cells -RANKL, as any acidic effects were localised to specific delineated sites underneath the osteoclast. However, pH levels were lowest at Day 8 where a significant increase in resorption pit formation was observed. The pH of cells +RANKL at Day 8 was pH 6.87. The pH required for optimal resorption activity of rat OC is pH 6.95[41]. Previous experimental work (Chapter 5, Section 5.4.2) investigating the effects of 15mM HCl addition to culture medium in the absence of cells recorded pH 7.52 after 8 days in culture. It was hypothesised that the metabolic activity of cells, acidic in nature, would further reduce culture medium pH. Cell metabolic activity produces CO_2 and H_20. The bicarbonate buffering system used in most culture media is standardized for either 5% or 10% CO_2. CO_2 is highly soluble in water and produces carbonic acid (H_2CO_3) that ionizes to H^+ and HCO_3^-. Increased cell activity increases the amount of CO_2 which produces more H^+ ions and acidifies the medium. This experiment has proven that addition of OC to acidified culture medium does further reduce pH (pH 7.52, previous cell free experiment to pH 6.87, current experiment). The optimal pH for OC formation is between pH 7.35-7.4 [134]. The recorded pH for Day 5 and 6 was pH 7.15 and 7.14 respectively. Not within, but close to the optimal range reported for OC formation the pH levels coincided with an increase in both TRAP activity (from Day 5) and OC number (from Day 6). The pH results of this experiment indicate that 15mM HCl added to culture medium provided sufficient acidification to firstly allow OC formation at a higher pH (pH ~7.1) followed by optimisation of activity and resorption pit formation at a lower pH (pH ~6.87) with time.

Mineral ion release into culture medium was assessed as a potential indicator of OC resorption based on the mechanism of OC-mediated resorption of CaP ceramics [146][121]. When an OC attaches to CaP, it secretes HCl [46][47] and enzymes [49][50][51] to digest the inorganic Ca and P ions forming a resorption pit under the actively resorbing OC. These ions are taken up by the OC, processed and released into the extracellular environment. As the inorganic mineral dissolves, the Ca^{2+} concentration within the microenvironment can increase from 8- 40 mmol/L (320 – 1600 mg/L) [46]. This local increase in Ca^{2+} concentration increases intracellular calcium promoting margin retraction and OC cell dehesion [112], ceasing resorption. After detachment of OC from the CaP the accumulated by-products of resorption are released into the extracellular environment. This sequence of events highlights how an increase in extracellular calcium, produced during active resorption, creates a negative feedback loop to prevent further resorption.

With that, as OC number and activity increases one would expect mineral ion release into culture medium to increase, to a point. This expectation was correct; mineral ion release into culture medium is time dependent and reflects the trends observed with TRAP activity and OC number. Ca ion concentration increased from Days 3 to 9 and a decreased at Day 12 whereas P ion concentration remained relatively stable from Days 3 to 9 and decreased at Day 12. Significant differences in Ca ($^{***}p<0.001$) and P ($^{**}p<0.01$) ion concentration in culture medium were observed between cell groups and no cell control groups.

Increases in extracellular calcium from 2-4mmol/L (80-160mg/L) have been shown to reduce resorption by 50% [113]. The Basal level of Ca in the culture medium was 40mg/L. Our results showed total Ca concentration in culture medium at Days 9 and 12 to be 66.8mg/L and 61.0mg/L respectively. Thus OC activity increased extracellular calcium concentration by 26.8mg/L and 21.0mg/L on Days 9 and 12

respectively. Considering extracellular calcium concentrations of 80-160mg/L can reduce resorption by 50% [113] one could presume that extracellular calcium concentrations of 40-80mg/L and 20-40mg/L could reduce resorption by 25% and 12.5% respectively. However our results did not show a reduction in resorption after 12 days with a total extracellular calcium concentration of 61.0mg/L. Therefore, one could reason that the reduction in extracellular Ca recorded by ICP-MS at Day 12 may directly correlate with the reduction in OC number and activity at Day 12. Ca ion concentration showed good positive correlation with OC count ($r = 0.708^{**}$) and negative correlation with pH ($r=-0.740^{**}$, as pH reduced Ca ion concentration increased) highlighting its potential as an outcome measure of OC resorption *in vitro*.

Percentage area resorbed significantly increased at Day 8 and maximum β-TCP surface resorption was ~20%. There was no significant difference in percentage area resorbed between Days 8 and 12 and standard deviation was large within each timepoint. Large standard deviations were observed for all timepoints. Higher magnification SEM micrographs illustrated that resorption pit depth appears to increase with time due to an increase in 'whiteness' of the pit areas with time. This may explain why no significant difference was observed when resorption results were presented as mean percentage area resorbed. The resorption pits may not increase in diameter but grow deeper and any changes may in fact be significant with depth. One could suspect the following sequence of events may have occurred after analysing results from the other outcome measures:

- At Day 8 resorption pits were formed
- At Day 10 resorption pits may have got deeper therefore changes in volume may be expected rather than increases in percentage area resorbed.

- At Day 12 OC number, TRAP activity and Ca release into culture medium had reduced indicating OC were less active and potentially dying off. Therefore resorption pit formation would have stabilised and no change in percentage area resorbed would be evident.

Log area had a moderate correlation with TRAP activity, OC count and Ca ion concentration ($r = 0.447^{*}$, 0.450^{*} and 0.599 respectively). Resorption pit area measurements may not be the most reliable outcome measure for assessing OC resorption especially considering the influence of heterogeneous cell spreading and OC formation across the sample, both of which increase standard error.

Statistical analysis showed ICP-MS, OC number and TRAP activity measurements to give a more conclusive picture than resorption pit area measurements alone. TRAP activity had good correlation with the number of TRAP positive OC ($r = 0.670^{**}$) and TRAP positive OC number had good correlation with Ca ion release ($r = 0.708^{**}$). All of which (TRAP activity, TRAP positive OC number and Ca ion release) had moderate correlation with log Area ($r = 0.447^{*}$, 0.450^{*} and 0.599 respectively).

6.5 Summary

The present study has shown that OC generated from RAW 264.7 cells attach to and degrade β-TCP *in vitro*. Evidence of TRAP activity, mineral release and resorption pit formation together provide the basis of this conclusion. Modifications to the OC assay were considered appropriate for the optimisation of RAW 264.7 OC formation and activity on β-TCP and RAW 264.7 OC were deemed as a suitable *in vitro* model for OC resorption of CaP materials.

The following highlights the suitability of the outcome measures assessed in this study as indicators of resorption *in vitro*:

- Total actin rings formed was an unsuitable measure of resorption due to variations in actin ring number and size giving inconclusive results.
- TRAP activity is a useful indicator of OC number and activity and is best used in conjunction with another outcome measure to indicate resorption.
- Total TRAP positive OC formed is a useful indicator of OC number and is a good to back up TRAP activity results but is very time consuming.
- SEM is an excellent indicator of resorption however is time consuming and pit area measurements alone are unsuitable.
- Ca ion release into culture medium is a suitable indicator of resorption.

ICP-MS, used to quantify Ca ion release into culture medium, coupled with TRAP activity measurements and SEM, may be an effective outcome measure for the prediction of OC mediated resorption of CaPs *in vitro*. ICP-MS and TRAP activity are:

- suitable indicators of OC resorption
- transferrable through a broad range of *in vitro* experiments
- cheaper and quicker than the current 2D methods used to quantify resorption.

In certain circumstances it may not be possible to measure pit area as an indicator of OC resorption for example, in porous scaffolds. ICP-MS and TRAP activity therefore have the potential for use as indicators of OC resorption for porous materials and materials with an uneven surface topography.

CHAPTER SEVEN

Overall discussion, conclusions and future work

7 Overall Discussion, Conclusions and Future work related to the *in vitro* OC resorption of CaP bone substitutes

7.1 Discussion and Conclusions

When identifying OC, many osteoclast related biochemical markers can be used. TRAP, located within the ruffled border of OC, is highly expressed by mature OC which stain positive for TRAP *in vitro* [141]. TRAP 5b enzyme can also be used as a marker of OC activity *in vitro* and is quantified by an ELISA specific for the OC-derived TRAP 5b enzyme [127]. RANK, a pre-OC membrane bound receptor, binds to its ligand RANKL (produced by osteoblasts and bone marrow stromal cells) promoting upregulation of RANK and subsequent OC differentiation [68]. RANK expression can be quantified *in vitro* by genomic analysis. Vitronectin ($\alpha v\beta 3$) and Calcitonin receptors [134] [147] are further biochemical markers present on OC. The vitronectin receptor binds to RGD-sequences on the surface of the material and the calcitonin receptor binds calcitonin. Binding of one or both of these receptors causes the attached OC to contract from the substrate ceasing further resorptive activity.

OC activity is generally characterised by cell morphology and cytoskeletal changes, biochemical tests and topographical assessment of the surface of the test material. Cell morphology changes include the fusion of mononuclear progenitors into multinucleated OC and the apico-basal morphology associated with OC attachment. Changes to the cytoskeleton are sealing zone and actin ring formation, both pre-requisites for OC resorption [31] [35] [147]. Colorimetric assays are used to quantify TRAP activity and calcium ion concentration *in vitro* [64] [95]. ICP-MS can also be used to quantify calcium ion concentration [64]. The trademark lacunae (pits) formed by active OC on the surface of the test material can be assessed by quantifying pit number, pit diameter, pit area and pit volume. Microscopical

techniques are used to quantify pit number (LM) pit area and diameter (LM, SEM) [67] and pit volume (confocal scanning laser microscopy, CSLM)[137]. Other methods used to assess changes in surface topography are XRD [148], surface profilometry [149] and micro computed tomography (micro-CT) [136]. XRD analysis allows quantification of any changes in crystallinity and phase composition of the material whereas surface profilometry detects changes in surface roughness of the material. Micro –CT performs 3D X-ray imaging of materials and is a non-destructive method whereas microscopy, XRD and surface profilometry are all destructive methods.

As there is no single method used to assess biomaterial resorption the 'pit assay' has become the most routinely used method. The rationale for the use of the pit assay as standard is that it allows visualisation and quantification of the physical work done by the OC (pit formation) and the microscopy equipment used to quantify pit measurements are readily available in most laboratories. Many of the methods listed above are suitable as indicators of OC resorption however, there has been no systematic attempt to identify an outcome measure of OC resorption that directly correlates with the current pit measurement assay and is transferrable through a broad range of *in vitro* experiments. The present work aimed to improve on the fundamental understanding of OC resorption of CaP bone substitutes *in vitro* by establishing the suitability of several outcome measures as possible indicators of osteoclastic resorption *in vitro*.

In order to be able to assess suitable outcome measures of OC resorption a number of objectives were set out;

- An OC assay needed to be developed to generate functionally active OC for the resorption of CaP materials *in vitro*.
- Various outcome measures needed to be assessed as indicators of resorption *in vitro*.

- Suitable outcome measures were required to directly correlate with resorp*tion pit* area measurements.
- Suitable outcome measures should be transferrable through a broad range of *in vitro* experiments.

A protocol by Osdoby et. al [56] was used as the foundation for establishing an experimental methodology for the generation of OC cells *in vitro*. The RAW 264.7 cell line was chosen on the basis that a cell line offers several advantages over primary cells; they are widely available, easy to culture, can be maintained indefinitely through sub-culturing, there is no inter-donor variability and there is no need to cull a live animal. RAW 264.7 cells also express M-CSF, unlike primary cells where M-CSF must be added to cultures to promote cell differentiation.

Initially, HA and β-TCP were chosen as CaP substrates and two different sintering temperatures were tested (1100°C and 1250°C) to determine if material phase composition and/or differences in sintering temperature affected OC formation and activity. The following outcome measures tested were; 1) SEM: to detect OC morphology and pit formation, 2) gravimetric analysis: to see if OC resorption could be related to a change in sample mass, 3) ICP-MS: to quantify mineral release from materials into culture and 4) TRAP 5b enzyme activity: to relate OC activity with resorption. The results from this study showed that few RAW 264.7 cells differentiated into OC and adhered to HA and β-TCP. The typical apico-basal morphology associated with active OC was not exhibited by the few remaining OC and they were smaller in size compared to those routinely cultured *in vitro* [124]. Early signs of pit formation were observed on both HA and β-TCP ceramics with superficial pit-like erosions on HA and more distinct pit-like areas on β-TCP. As previously discussed, controversial results for the resorption of HA *in vitro* have been reported with some authors observing OC resorption of HA [95][64] and others not [96]. It has been well documented that the solubility of ceramics can influence

their resorption *in vitro* [95][64][96]. Samples sintered at 1250°C had higher calcium ion levels in the culture medium than their 1100°C counterparts. In theory CaPs sintered at higher temperatures should be more thermodynamically stable than those sintered at lower temperatures and exhibit lower ion levels in the culture medium. For HA and β-TCP, phase changes can occur after a threshold sintering temperature has been reached (HA > β-TCP > α-TCP) [22]. α-TCP is more soluble than β-TCP thus if changes in phase composition did occur, this may have increased the solubility of the material and may help explain the increased calcium ion concentration in the culture medium for 1250°C samples. Also, since higher local Ca^{2+} concentrations inhibit OC resorption [109] then perhaps the theoretically more soluble 1100°C materials inhibited OC activity because they had dissolved more readily. To give a greater understanding of the material phase composition of the different samples XRD analysis could be conducted. The use of surface profilometry was considered for CaP characterisation but after discussion with experienced users we realised our surfaces would be too rough, only a small sample size can be analysed and it would be destructive for samples containing cells. Gravimetric analysis was deemed unsuitable as a measure of OC resorption on the basis of large Inter-group and intra-group variation for both materials. Potential sources of variation with this outcome measure could be as a result of the degree of compaction of the sintered sample (some samples could be more porous than others) or the variation in evaporation of HMDS from the surface of the sample. There was no statistical correlation with percentage mass change and cell number for HA or β-TCP; $p = 0.406$ or any statistical differences between those with cells compared to no cell control. ICP-MS showed good correlation with TRAP 5b enzyme activity indicating that Ca ion concentration could be related to cellular activity and showed potential as a measure of resorption. This work highlighted the potential of RAW 264.7 cells to degrade CaP ceramics *in vitro* but it also emphasized a limitation of the current 2D method (pit area). It was hard to decipher osteoclast specific resorption

pits from anomalies on the surface of the material. In order to ascertain a pit from a pore or surface defect a smoother surface finish of the CaP is ideally required.

In an attempt to culture adequate numbers of functionally mature OC, a comparative study was conducted on the differentiation and function of RAW 264.7 cells with primary OPC. Dense, lightly polished β-TCP was used to obtain a smoother surface finish for easier detection and quantification of resorption pits formed. Most differentiation was observed at a RANKL dose of 20ng/mL for both OPC and RAW 264.7 cells. It was also noted that a cell culture period of more than 6 days should be employed as OPC number had not yet reached their maximum after 6 Days in culture. Following the preliminary experiment, modifications to the OC assay included a RANKL dose of 20ng/mL and a cell culture period of 10 days. Additional modifications to the OC assay included the use of α-MEM instead of DMEM and the addition of 15mM HCl to cultures on Day 6 to optimise the acidic conditions required for OC formation and activity *in vitro* [41]. To ascertain whether differences in sintering procedure affected the development of OC on CaP materials, dense β-TCP (sintered at 1100°C) was either analysed as such or heat treated (calcined) at 500°C for 24h to remove any surface defects. The following outcome measures tested were: TRAP positive OC number and activity, actin ring formation, percentage surface area and surface properties were determined by SEM.

RAW 264.7 cells produced more mature OC than OPC when cultured in the absence of CaP (Chapter 4 Exp A) after 6 days. However, marginally more OPC were present at Day 6 and significantly more OPC at Day 10 compared to RAW 264.7 cells when cultured on β-TCP. It is known that OC prefer rough surfaces to smooth surfaces [131][31][76] therefore the presence of a rougher β-TCP substrate should not reduce OC formation compared to smoother tissue culture plastic. Perhaps a combination of the interaction of cell culture medium with the surface of the β-TCP and the difference in surface energy of tissue culture plastic compared to β-TCP may

have influenced RAW 264.7 OC formation and adhesion. Surface energy and surface chemistry have been shown to affect cell adhesion and cell spreading respectively [132]. Also, RAW 264.7 OC were larger (100-500 micron) than OPC (50-100micron) and thus fewer RAW 264.7 OC could theoretically fit into the field of view during quantification by light microscopy potentially. This work highlights how a longer culture period gave a more conclusive story on the differentiation and function of RAW 264.7 OC versus OC derived from OPC.

As previously discussed RAW 264.7 cells are quick to culture and many cultures can be obtained from a single vial. However they are not without their drawbacks. Cell lines may not react in the same way as primary cells and should be used for preliminary studies or indeed in parallel to primary cell studies in order to give a more comprehensive assessment of *in vitro* OC resorption studies. RAW 264.7 OC first appear around Days 3-4 and become plentiful by Days 5-6 before dying off. The number of OC formed varies considerably with both the number of cells seeded and the passage number of the RAW 264.7 cells used. Early and late passages generally produce low numbers of OCs with optimal results achieved using cells from passages 4–18 [150]. All of the RAW 264.7 studies in this body of work were not conducted using the same passage number and this in itself introduces a limitation for the reproducibility of the reported experimental work.

OC number aside, OC generated from OPC and RAW 264.7 cells displayed the same general trends when assessing OC differentiation and function with the exception of resorption pit shape and actin ring number. There was a ~5 fold increase in TRAP positive OC for OPC and a ~3 fold increase for RAW 264.7 from Day 6-10. No resorption pits were formed at Day 6 followed by formation and increase in resorption pit formation up to Day 10. For actin ring formation: OPC Mean AR count increased from Day 4-8 and decreased at Day 10 whereas RAW 264.7 OC Mean AR count had a marginal increase from Day 6 to Day 10. For resorption pit

shape: OPC resorbed in a linear fashion revealing resorption trails whereas RAW 264.7 resorption appeared more static forming distinct circular resorption pits. Although the shape of the resorbed areas differed with cell type the shape reported was the same as described in [123] for both RAW 264.7 and OPC. Addition of 15mM HCl and the use of α-MEM may have played a role in acidifying the OC extracellular environment in favour of OC formation and activity and using a dense lightly polished substrate made it easier to see resorption pits on flatter surfaces.

Calcination of dense β-TCP had no effect on resorption by the OPC or RAW 264.7 OC *in vitro* or on OC formation and activity. Others [128] reported a significant reduction in OPC TRAP activity for non-calcined β-TCP compared to calcined β-TCP after 5 days in culture. The study in [128] proposed that a change in surface dissolution capacity of the calcined and non-calcined β-TCP may have altered the OC adhesion and/or migration mechanism. No quantification of OC resorption on calcined and non-calcined β-TCP was reported in the study. The area of sintering temperature and calcination was not to be investigated further. In order to prioritise assay development and to fully interpret RAW 264.7 cell results, more timepoints were required to investigate RAW 264.7 cells more comprehensively as an *in vitro* model for the OC resorption of CaP materials.

A series of experiments were set up to justify new protocol modifications. Namely, the use of 20ng/mL RANKL, the addition of 15mM HCl to cell cultures and the timepoint at which HCl was added to cultures. A comparative study was conducted to assess RANKL dose and supplier on the differentiation of OC. PeproTech RANKL indicated that there was more OC differentiation compared to R&D Systems RANKL due to the increased TRAP activity results at lower concentrations. When trying to quantify resorption, there is a need for standardisation of reference materials between laboratories. Experimental results yielded from studies using different RANKL doses and/or RANKL from different suppliers could potentially

yield false or misleading results i.e. would results for the quantification of resorption for a given RANKL dose from one supplier verses another supplier be comparable? As discussed above, this work found discrepancies between brands. Ideally the test material in question should be tested with the same reference materials before introducing any other variables. This highlights a limitation of *in vitro* testing in general and in the context of this work that TRAP activity of RANKL differentiated OC is useful as an indicator of OC activity for preliminary screening but further testing of promising materials is necessary, ideally *in vivo*.

A cell free experiment was set up to compare the surface of β-TCP discs before and after addition of HCl in a cell free environment to ensure that any pits observed previously on β-TCP discs were a result of cell mediated action rather than by dissolution as a direct effect of the presence of HCl. The element release profiles for 15mM HCl 5% CO_2 (modified protocol) and 0mM HCl 5% CO_2 (initial protocol) showed very little difference in Ca ion concentration after Day 3. SEM micrographs of the β-TCP surfaces showed that no pits or pit-like areas were present after incubation with or without HCl. Thus it was concluded that the pits obtained whilst using 15mM HCl at 5% CO_2 were cell-mediated rather than by dissolution mechanisms caused by the presence of HCl.

Metabolic activity of cells is acidic in nature and further reduces culture medium pH. The optimal pH for OC formation is between pH 7.35-7.4 [134]. pH results indicated that 15mM HCl added to culture medium at 5% CO_2 may provide sufficient acidification to firstly allow OC formation at a higher pH (pH ~7.4) followed by optimisation of activity and resorption pit formation at a lower pH with time. It must be noted that the most acidic level recorded at Day 10 was still higher than the recommended optimal pH 6.95 for resorption pit formation [41]. In general, the recorded pH values were higher than suggested in [41]. This was due to the fact that this particular experiment was conducted in the absence of cells and some degree

of alkalinity was expected. Secondly, the sampling method used for pH involved placing the medium in eppendorfs and this in itself may have increased pH during sample transfer (exposed to atmospheric conditions (~0.5% CO_2) compared to those in the incubator (5%CO_2).

In a cell free environment, increased acidification increased Ca ion concentration. For all %CO_2 groups there was a general decrease in Ca ion concentration with time, indicative of reprecipitation onto the β-TCP disc [64]. This highlights a limitation of Ca ion concentration analysis as a single measure of OC resorption of CaP materials *in vitro*. CaP ceramics with varying CaP ratios will reprecipitate at different rates therefore the level of Ca ion concentration in the medium may not be fully indicative of total resorption. Different CaP materials will have different amounts of calcium per unit volume. Therefore when interpreting OC resorption for various CaP materials, reprecipitation could confound results.

Final outcome measures assessed were Ca and P mineral release into cell culture medium, OC number and activity, actin ring formation and pH. In order to accurately correlate pit area with the alternative outcome measures, the assay was performed using dense β-TCP free from surface imperfections. All measured outcomes varied significantly with time and reflected the natural cycle of OCs in culture as they were formed from monocyte precursors, increased in activity and then became exhausted and apotosed [151]. OC formation was established by the expression of TRAP and the formation of actin rings within multinucleated cells.

Active OC have been shown to express higher levels of TRAP activity compared to inactive OC [145]. The number of TRAP positive OC correlated with TRAP activity however RAW 264.7 cells without RANKL addition displayed similar TRAP activity to cells with RANKL at Days 4 and 12. This basal level of activity must be taken into consideration when using TRAP activity as an indication of OC function.

From Day 6 to Day 10 there was a time dependent increase in TRAP activity of which was significantly higher than the basal activity. Therefore TRAP activity is a useful indicator of OC activity provided the basal level of activity is accounted for.

When an OC is polarised on a substrate surface a sealing zone forms containing a dense ring of actin. The presence of the actin ring is indicative of the resorptive phase of the OC [4,36] therefore it was presumed that actin ring formation could also be a potential measure of OC resorption *in vitro*. However, actin ring formation did not follow the same trends observed with TRAP positive OC count and TRAP activity results. One may expect to see an increase in actin ring formation with an increase in OC number but this was not the case. Observations from fluorescence microscopy indicated that the size and shape of actin rings changed with time, generally increasing in size. This resulted in a decreasing ratio of actin rings to TRAP positive OC. Although the number of actin rings did correlate with TRAP activity, this was only of moderate strength and the variation in this outcome measure suggests that it would not be suitable for use for *in vitro* biomaterial testing.

The pH required for optimal resorption activity of rat OC is pH 6.95 [41]. Previous experimental work investigating the effects of 15mM HCL addition to culture medium in the absence of cells recorded pH 7.52 after 8 days in culture. It was hypothesised that the metabolic activity of cells, acidic in nature, would further reduce culture medium pH. Certainly OC exhibit a change in pH when they are in their active state although this change is within a localised region beneath the cell delineated by the sealing zone and ruffled border [45][47]. Addition of OC to acidified culture medium further reduced the pH and coincided with an increase in both TRAP activity (from Day 5) and OC number (from Day 6). Furthermore, a reduction in pH was correlated to an increase in OC number ($r=0.420$) and an increase in Ca ion concentration in the medium ($r=0.740$) which might suggest that this could be used as part of a suite of measures to indicate resorption.

Mineral ion release into culture medium was assessed as a potential indicator of OC resorption based on the mechanism of OC-mediated resorption of CaP ceramics [121]. When an OC attaches to CaP, it secretes HCL [46] and enzymes [49] [50] [51] to digest the inorganic Ca and P ions forming a resorption pit under the actively resorbing OC. These ions are taken up by the OC, processed and released into the extracellular environment. As the inorganic mineral dissolves, the Ca^{2+} concentration within the microenvironment can increase from 8- 40 mmol/L (320–1600 mg/L) [46]. This local increase in Ca^{2+} concentration increases intracellular calcium, promoting margin retraction and OC cell dehesion [112], ceasing resorption. After detachment of OC from the CaP, the accumulated by-products of resorption are released into the extracellular environment. With that, as OC number and activity increases one would expect mineral ion concentration in the culture medium to increase. It was found that, Ca ion concentration in culture medium was time dependent and reflected the trends observed with TRAP activity and OC number, correlating well with both, in addition to change in pH. P ion release was slower to increase and was not elevated until Day 9 although it was present in greater amounts in medium from cultures with cells compared to without cells. Perhaps it was this lag in P release which led to the surprising finding that P ion concentration was negatively correlated with TRAP activity and resorption pit area.

Each of the outcome measures above are considered as adjuncts to the "gold standard", the resorption pit assay, which other results must be compared. The results showed that the percentage area resorbed significantly increased at Day 8 and the maximum β-TCP surface resorption was ~20%. Although no significant difference in percentage area resorbed was found beyond Day 8 standard deviations were large and SEM analysis suggested that resorption pit depth increased with time that was not reflected in area measurements. Even given this variability, resorption pit area still showed a moderate correlation with TRAP activity and OC count ($r = 0.447^{*}$ and

0.450* respectively) which provides strong evidence for the use of these outcome measures as surrogate endpoints of resorption. Volumetric measurements of the resorption pits formed may have been preferable and could have proved more sensitive and more strongly correlated to other measures, however, analysis of this kind requires specialised and expensive equipment which was not available for this study [136][137][138][139].

The results of this study would suggest that TRAP activity and OC number, are suitable to measure resorption of a ceramic substrate *in vitro* and this could be strengthened by the addition of Ca ion concentration and pH. Caution must be taken however, to control for basal levels of TRAP activity in monocytic cells and to ensure that results are taken during the peak of OC activity. If all four outcome measures indicate similar results, this should provide robust evidence that could replace the more accepted resorption pit assay in circumstances where quantification of pits is not possible, for example when testing resorbability of 3D scaffolds *in vitro*.

7.2 Overall Conclusions

There are many limitations associated with the current methods used to assess bioresorption of CaP materials *in vitro*. Traditional methods used to assess bioresorption use microscopy techniques and quantify resorption on a 2D basis. A 3D quantification of bioresorption is available but requires specialised, expensive equipment. Pit area measurements (2D) have become common practice when assessing biomaterial resorption *in vitro* but it is not a precise indicator of resorption; variations in pit depth are not taken into consideration and it is unsuitable for use on porous materials or materials with rough surfaces. There has been no systematic attempt to identify an outcome measure of bioresorption that directly correlates with pit area measurements and is transferrable through a broad range of *in vitro* experiments.

The following conclusions can be drawn from the findings of this research:

- The RAW 264.7 cell line is a suitable model of *in vitro* resorption of CaP.
- A dense, flat, polished β-TCP surface is the most suitable substrate for assessing resorption by RAW 264.7 OC *in vitro*.
- Calcination of β-TCP surface had no effect on OC formation and resorption *in vitro*.
- A RANKL dose of 20ng/mL (Peprotech) was the optimal dose for OC differentiation of RAW 264.7cells *in vitro*.
- A combination of using alpha-MEM and the addition of 15mM HCl to cell culture medium from Day 3 acidified the cell culture medium to a near optimal pH level for OC formation *in vitro*.
- The addition of 15mM HCl to cell culture medium had no effect on β-TCP surfaces after culturing in a cell free environment for 12 days.

- The optimal cell culture period for the formation of RAW 264.7 OC and resorption of β-TCP is between 9 and 12 days.
- Actin ring formation and gravimetric analysis are unsuitable as indicators of OC resorption *in vitro* as they provide inconclusive results and do not correlate with standard pit area measurements.

  The use of ICP-MS, to detect calcium ion concentration in cell culture medium, coupled with TRAP activity measurements are the most suitable indicators of OC resorption *in vitro* that correlate with standard pit area measurements and have potential for use as a measure of resorption throughout a range of *in vitro* experiments.

In summary, this research has established several suitable outcome measures of bioresorption *in vitro* that correlate with pit area measurements. The method developed shows promise as an *in vitro* assay of resorption that could be transferrable through a range of experiments, proving invaluable for improving the fundamental understanding of OC resorption of ceramic biomaterials.

7.3 Future Direction

It is known that OC prefer rough surfaces to smooth surfaces when resorbing [76][152][131][31]. Smooth substrates produce small, unstable actin rings that last ~8minutes whereas rough substrates produce large, peripheral more stable actin rings lasting ~1hour [76]. Surface nano-topography both initiates resorptive actin ring formation and regulates resorptive ring dynamics [76]. For OC to resorb, the OC sealing zone must be coherent and stable for extended periods of time. This can be controlled by introducing three-dimensional ridges (increasing surface roughness) which act like barriers to help prevent OC sealing zones from expanding [152].

Previous studies have been carried out on different substrates such as; glass, HA, calcium carbonate (CC), β-TCP, ion-coated substrates and ceramic composites with varying substrate ratios, all having subsequent differences in surface roughness [96][64][95][74]. However, less work has been carried out using a single material with the same material properties with the only variable being different surface roughness profiles - which is what I would propose. It is difficult to see resorption on a rougher surface; however this body of work has proved that ICP-MS is a suitable outcome measure as an indicator of OC resorption *in vitro*.

After reviewing the literature I feel that a feasible next step for progression of this work would be to change the surface roughness whilst keeping all other material parameters constant. My proposal would be to use polished, unpolished, chemically-etched and pre-resorbed samples. Surface roughness of polished samples could be further subdivided using varying degrees of abrasive paper. I think it would be interesting to validate the use of ICP-MS as an indicator of resorption *in vitro* by using it to assess variations in CaP bone substitutes.

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